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PROVISIONAL APPLICATION COVER SHEET

To the Commissioner of Patents and Trademarks
Washington, DC 20231
This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

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TITLE OF THE INVENTION (280 characters max)

ACYLUREA CONNECTED AND SULFONYLUREA CONNECTED HYDROXAMATES

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification Number of Pages 44

METHOD OF PAYMENT (check one)

☒ A check or money order is enclosed to cover the Provisional filing fees ☒ The Commissioner is hereby authorized to charge any additional fees and credit any overages to Deposit Account Number: 04-1577	Provisional filing fee amount(s)	$ 160.00

Respectfully submitted,

Signature: ____________________ Date: October 27, 2003

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ACYLUREA CONNECTED AND SULFONYLUREA CONNECTED HYDROXAMATES

FIELD

Embodiments are disclosed of hydroxamate compounds which are inhibitors of histone deacetylase. More particularly, there are disclosed certain biaryl containing compounds and methods for their preparation. These compounds are useful as medicaments for the treatment of proliferative diseases.

BACKGROUND

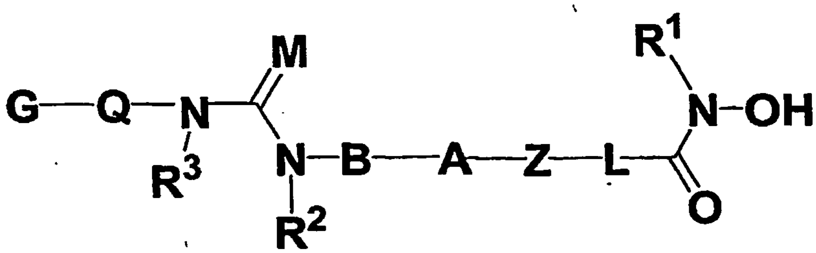
Local chromatin architecture is generally recognized as an important factor in the regulation of gene expression. The architecture of chromatin, a protein-DNA complex, is strongly influenced by post-translational modifications of the histones which are the protein components. Reversible acetylation of histones is a key component in the regulation of gene expression by altering the accessibility of transcription factors to DNA. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression [1,2]. In normal cells, histone deacetylase (HDACs) and histone acetyltransferase together control the level of acetylation of histones to maintain a balance. Inhibition of HDACs results in the accumulation of hyperacetylated histones, which results in a variety of cellular responses, such as apoptosis, necrosis, differentiation, inhibition of proliferation and cytostasis.

Inhibitors of HDAC have been studied for their therapeutic effects on cancer cells. For example, suberoylanilide hydroxamic acid (SAHA) is a potent inducer of differentiation and/or apoptosis in murine erythroleukemia, bladder, and myeloma cell lines [3,4]. SAHA has been shown to suppresses the growth of prostate cancer cells *in vitro* and *in vivo* [5]. Other inhibitors of HDAC that have been widely studied for their anti-cancer activities are trichostatin A (TSA) and trapoxin B [6,7]. Trichostatin A is a reversible inhibitor of mammalian HDAC. Trapoxin B is a cyclic tetrapeptide, which is an irreversible inhibitor of mammalian HDAC. However, due to the in vivo instability of these compounds they are less desirable as anti-cancer drugs. Recently, other small molecule HDAC inhibitors have become available for clinical evaluation [8]. Additional HDAC inhibiting compounds have been reported in the literature [Bouchain G. et al, J. Med. Chem., 46, 820-830 (2003)] and patents [WO 03/066579A2]. HDAC inhibitors have been reported to interfere with neurodegenerative processes, for instance, HDAC inhibitors arrest polyglutamine-dependent neurodegeneration [Nature, 413, 18 October, 2001].

Despite recent advances, there remains a need for an active compound with desirable activity, solubility, and metabolic properties that is suitable for treating cancerous tumors.

SUMMARY

There are disclosed histone deacetylase inhibitor compounds having the formula (I):



Formula (I)

wherein

- R^1 is selected from H, C_1 -C_6 alkyl, acyl;
- L is a single bond or is a C_1-C_5 hydrocarbon chain which may contain 0 to 2 double bond or triple bond or one double bond and one triple bond, the chain may be optionally interrupted by -O-, -S-, -S(O)- and -S(O)$_2$-, and unsubstituted or substituted with one or more substituents independently selected from the group consisting of C_1-C_4 alkyl;
- Z is single bond or selected from O, S, S(O), S(O)$_2$, etc.
- A is single bond or a ring which is selected from arylene, heteroarylene, cycloalkylene and heterocycloalkylene which could be further optionally substituted by X and Y.
- B is single bond or a ring which is selected from arylene, heteroarylene, arylalkylene, heteroarylalkylene, C_1-C_3 alkylene, heteroalkylene, cycloalkylene and heterocycloalkylene which could be further optionally substituted by X and Y.
- M is selected from O, S, NH, NR^4, NOH and NOR^4.
- R^2 and R^3 are the same or different and independently H, C_1 -C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 -C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), hydroxyl, hydroxyalkyl, alkoxy, amino, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR^4, -CONHR^4, -NHCONHR^4, C(=NOH)R^4, and acyl;
- Q is selected from -S(O)$_2$-, -CO- and –C(=S)-.
- G is selected from aryl, heteroaryl, alkyl, cycloalkyl, heterocycloalkyl, arylalkyl and heteroarylalkyl; they also could be further substituted by X, Y, R^4, hydroxyl, hydroxyalkyl, alkoxy, amino, al-

kylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR^4, -C(O)OH, -SH, -CONHR^4, -NHCONHR^4, and C(=NOH)R^4.

- X and Y are the same or different and independently selected from hydrido, halo, C_1 -C_4 alkyl, such as CH_3 and CF_3, NO_2, OR^4, SR^4, C(O)R$_5$, CN, and NR^6 R^7 ;
- R^4 is selected from C_1-C_4 alkyl, heteroalkyl, aryl, heteroaryl, acyl;
- R^5 is selected from C_1-C_4 alkyl;
- R^6 and R^7 are the same or different and independently selected from hydrido, C_1-C_6 alkyl, C_4-C_9 cycloalkyl, C_4-C_9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

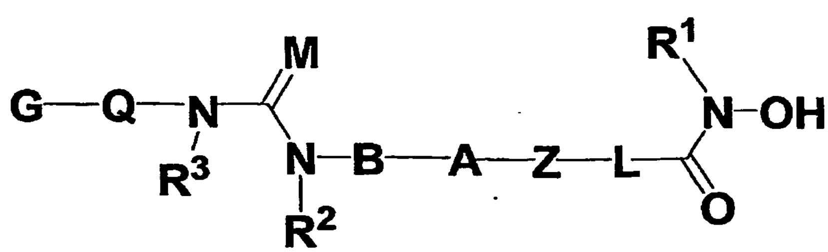
or a pharmaceutically acceptable salt thereof.

In addition to compounds of Formula I, the embodiments disclosed are also directed to pharmaceutically acceptable salts, pharmaceutically acceptable prodrugs, and pharmaceutically active metabolites of such compounds, and pharmaceutically acceptable salts of such metabolites. Such compounds, salts, prodrugs and metabolites are at times collectively referred to herein as "HDAC inhibiting agents". The embodiments disclosed also relate to pharmaceutical compositions each comprising a therapeutically effective amount of a HDAC inhibiting agent of the embodiments described with a pharmaceutically acceptable carrier or diluent for treating cellular proliferative ailments. The term effective amount as used herein indicates an amount necessary to administer to a host to achieve a therapeutic result, e.g., inhibition of proliferation of malignant cancer cells, benign tumor cells or other proliferative cells.

Other embodiments describe use of the above compounds and compositions to treat diseases or to modify deacetylase activity.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

There are disclosed hydroxamate compounds, for example acylurea or sulfonylurea containing hydroxamic acid in one of the substituents, that may be inhibitors of deacetylases, including but not limited to inhibitors of histone deacetylases [2]. The hydroxamate compounds may be suitable for treating tumors, including cancerous tumors. The hydroxamate compounds of the present embodiments have the following structure (I):



Formula (I)

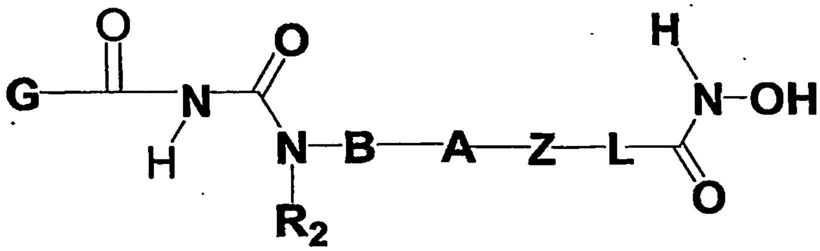
wherein

- R^1 is selected from H, C_1 -C_6 alkyl, acyl;
- L is a single bond or is a C_1-C_5 hydrocarbon chain which may contain 0 to 2 double bond or triple bond or one double bond and one triple bond, the chain may be optionally interrupted by -O-, -S-, -S(O)- and -$S(O)_2$-, and unsubstituted or substituted with one or more substituents independently selected from the group consisting of C_1-C_4 alkyl;
- Z is single bond or selected from O, S, S(O), $S(O)_2$, etc.
- A is single bond or a ring which is selected from arylene, heteroarylene, cycloalkylene and heterocycloalkylene which could be further optionally substituted by X and Y.
- B is single bond or a ring which is selected from arylene, heteroarylene, arylalkylene, heteroarylalkylene, C_1-C_3 alkylene, heteroalkylene, cycloalkylene and heterocycloalkylene which could be further optionally substituted by X and Y.
- M is selected from O, S, NH, NR^4, NOH and NOR^4.
- R^2 and R^3 are the same or different and independently H, C_1 -C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 -C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), hydroxyl, hydroxyalkyl, alkoxy, amino, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR^4, -$CONHR^4$, -$NHCONHR^4$, C(=NOH)R^4, and acyl;
- Q is selected from -$S(O)_2$-, -CO- and –C(=S)-.
- G is selected from aryl, heteroaryl, alkyl, cycloalkyl, heterocycloalkyl, arylalkyl and heteroarylalkyl; they also could be further substituted by X, Y, R^4, hydroxyl, hydroxyalkyl, alkoxy, amino, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR^4, -C(O)OH, -SH, -$CONHR^4$, -$NHCONHR^4$, and C(=NOH)R^4.
- X and Y are the same or different and independently selected from hydrido, halo, C_1 -C_4 alkyl, such as CH_3 and CF_3, NO_2, OR^4, SR^4, C(O)R^5, CN, and NR^6R^7;
- R^4 is selected from C_1-C_4 alkyl, heteroalkyl, aryl, heteroaryl, acyl;
- R^5 is selected from C_1-C_4 alkyl;
- R^6 and R^7 are the same or different and independently selected from hydrido, C_1-C_6 alkyl, C_4-C_9 cycloalkyl, C_4-C_9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

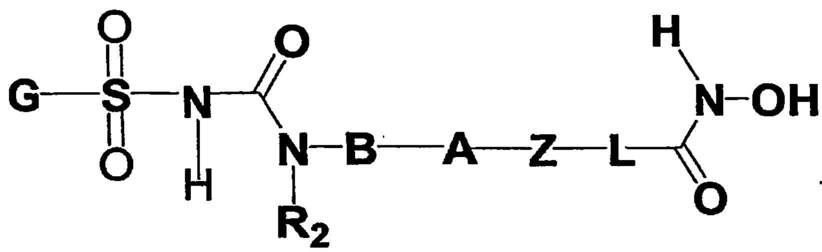
In further embodiments there is disclosed compound of Formula (I) wherein each of R^1, R^3, X and Y is H.

In further embodiments there is disclosed compound of formula (Ia) wherein $R^1 = R^3 = H$; R^2, X, Y, Z, A, B, R^3 and R^4 are the same as for Formula (I).



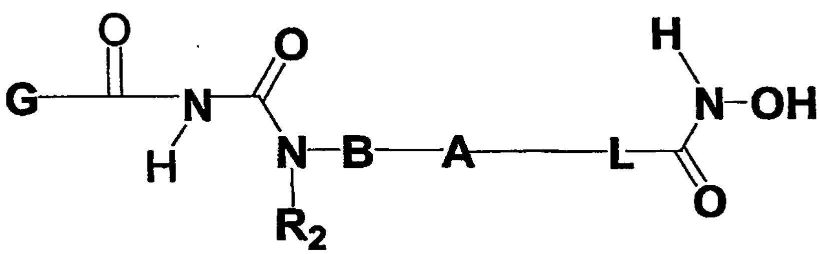
Formula (Ia)

In further embodiments there is disclosed a compound of formula (Ib) wherein $R^1 = R^3 = H$; R^2, X, Y, Z, A, B, R^3 and R^4 are the same as for formula (I).



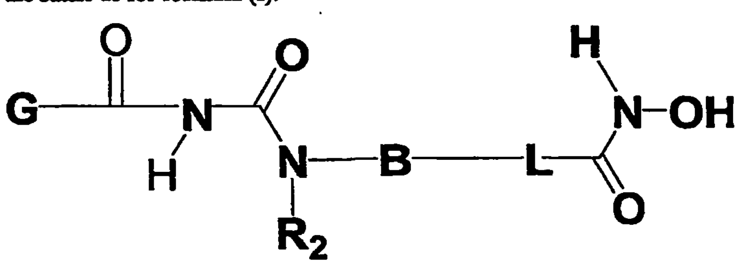
Formula (Ib)

In further embodiments there is disclosed a compound of formula (Ic) wherein R^2, X, Y, L, A, B, G, R^3 and R^4 are the same as for formula (I).



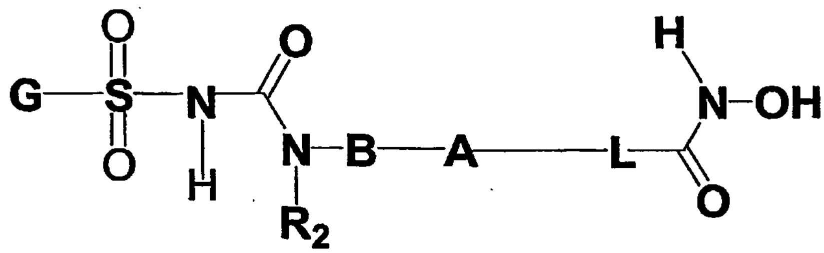
Formula (Ic)

In further embodiments there is disclosed a compound of formula (Id) wherein R^2, X, Y, L, B, G and R^4 are the same as for formula (I).



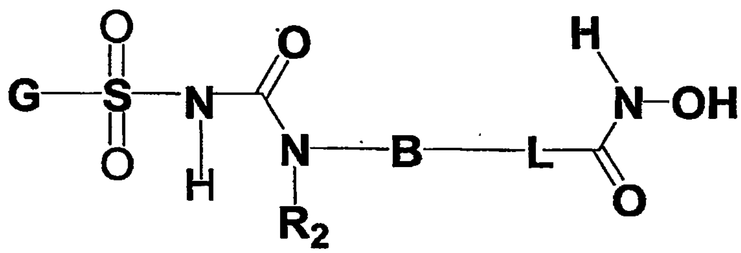
Formula (Id)

In further embodiments there is disclosed a compound of formula (Ie) wherein R^2, X, Y, L, A, B, G, L and R^4 are the same as for formula (I).



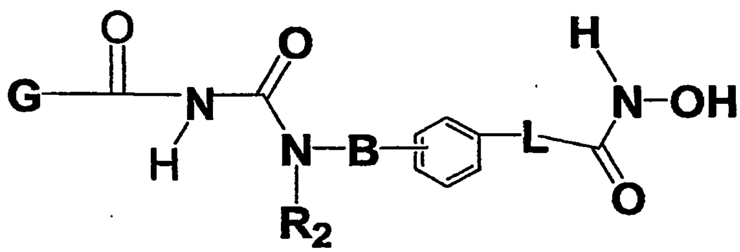
Formula (Ie)

In further embodiments there is disclosed a compound of formula (If) wherein R^2, X, Y, L, B, G, L and R^4 are the same as for formula (I).



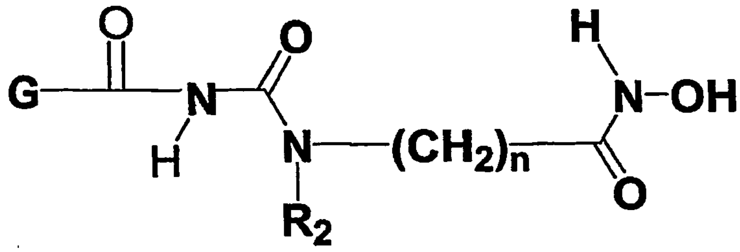
Formula (If)

In further embodiments there is disclosed a compound of formula (Ig) wherein B is a single bond or CH_2, L is a single bond or selected from CH_2, CH_2CH_2, –CH=CH-, -C-triple bond-C-, R^2 is selected from H, alkyl, arylalkyl, arylheteroalkyl, heteroarylalkyl, heteroarylheteroalkyl. B is attached to meta or para position of phenylene against L. G is selected from aryl, heteroaryl, alkyl and alkoxyalkyl.



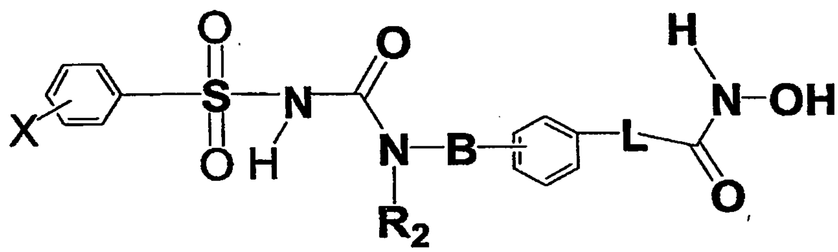
Formula (Ig)

In further embodiments there is disclosed a compound of formula (Ih) wherein n is an integer from 1 to 8; R^2 is selected from H, alkyl, arylalkyl, arylheteroalkyl, heteroarylalkyl, heteroarylheteroalkyl; G is selected from aryl, heteroaryl, alkyl and heteroalkyl.



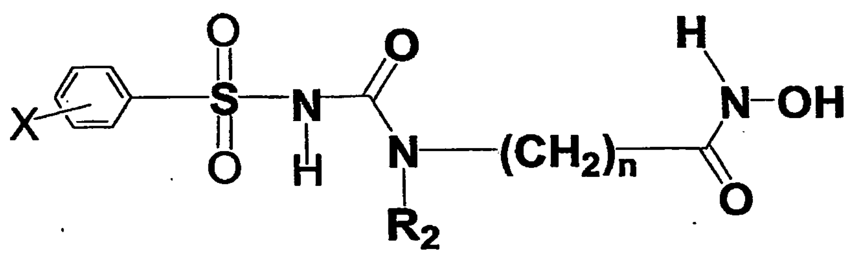
Formula (Ih)

In further embodiments there is disclosed a compound of formula (Ii) wherein B is a single bond or CH_2, L is a single bond or selected from CH_2, CH_2CH_2, –CH=CH-, -C-triple bond-C-, R_2 is selected from H, alkyl, arylalkylaryl, heteroalkyl, heteroarylalkyl, heteroarylheteroalkyl. X is selected from H, halo, C_1-C_4 alkyl, alkoxy, alkylamino; B is attached to meta or para position of phenylene against L.



Formula (Ii)

In further embodiments there is disclosed a compound of (Ij) wherein n is an integer from 1 to 8, X is selected from H, halo, C_1-C_4 alkyl, alkxoy, alkylamino.



Formula (Ij)

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (I) to treat proliferative diseases, including cancerous tumors.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ia) to treat proliferative diseases, including cancerous tumors.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ib) to treat proliferative diseases, including cancerous tumors.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ig) to treat proliferative diseases, including cancerous tumors.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ih) to treat proliferative diseases, including cancerous tumors.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (I) to modify deacetylase activity, preferably histone deacetylase activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ia) to modify deacetylase activity, preferably histone deacetylase activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ib) to modify deacetylase activity, preferably histone deacetylase activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ig) to modify deacetylase activity, preferably histone deacetylase activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ih) to modify deacetylase activity, preferably histone deacetylase activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ib) to modify histone deacetylase activity, preferably HDAC8 activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ig) to modify histone deacetylase activity, preferably HDAC1 and HDAC8 activity.

In further embodiments there is disclosed the use of a pharmaceutical composition of compound of formula (Ih) to modify histone deacetylase activity, preferably HDAC1 and HDAC8 activity.

or a pharmaceutically acceptable salt thereof.

In further embodiments, there are disclosed pharmaceutical compositions comprising the compounds disclosed herein.

As used herein, the term unsubstituted means that there is no substituent or that the only substituents are hydrogen.

The term "Halogen" represents chlorine, fluorine, bromine or iodine. The term "halo" represents fluoro, chloro, bromo and iodo.

The term "Alkyl" refers to a straight or branched C_1 -C_6 alkyl, unless otherwise noted. Examples of suitable straight and branched C_1 -C_6 alkyl substituents include methyl, ethyl, n-propyl, 2-propyl, n-butyl, sec-butyl, t-butyl, hexyl, and the like. When the alkyl is divalent it has been referred to as "alkylene" in this application.

The term "Acyl" denotes a radical provided by the residue after removal of hydroxyl from an organic acid, examples of such radical being acetyl, benzoyl, phenylacetyl and phenoxyacetyl.

The term "Cycloalkyl" refers to a saturated or partially saturated, monocyclic or fused or spiro polycyclic, carbocycle from C_3 -C_9 per ring, such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and the like, unless otherwise specified. When the cycloalkyl is divalent it has been referred to as "cycloalkylene" in this application.

The above discussion of alkyl and cycloalkyl substituents also applies to the alkyl portions of other substituents, such as without limitation, alkoxy, alkyl amines, alkyl ketones, arylalkyl, heteroarylalkyl, alkylsulfonyl and alkyl ester substituents and the like.

The term "Heterocycloalkyl" refers to a 3 to 9 membered aliphatic rings, such as 4 to 7 membered aliphatic rings, containing from one to three heteroatoms selected from nitrogen, sulfur, oxygen. Examples of suitable heterocycloalkyl substituents include pyrrolidyl, tetrahydrofuryl, tetrahydrothiofuranyl, piperidyl, piperazyl, tetrahydropyranyl, morphilino, 1,3-diazapane, 1,4-diazapane, 1,4-oxazepane, and 1,4-oxathiapane. When the heterocycloalkyl is divalent it has been referred to as "heterocycloalkylene" in this application.

The term "Heteroalkyl" refers to a straight- or branched-chain alkyl group having from 2 to 12 atoms in the chain, one or more of which is a heteroatom selected from S, O, and N. Exemplary heteroalkyls include alkyl ethers, secondary and tertiary alkyl amines, alkyl sulfides, and the like. When the heteroalkyl is divalent it has been referred to as "heteroalkylene" in this application.

The term "Aryl" refers to a monocyclic, or fused polycyclic, aromatic carbocycle (ring structure having ring atoms that are all carbon) having from 5 to 12 atoms per ring. Examples of aryl groups include phenyl, naphthyl, and the like. When the aryl ring is divalent it has been referred to as "arylene" in this application.

The term "Heteroaryl" refers to a monocyclic, or fused polycyclic, aromatic heterocycle (ring structure having a 5 to 7 member aromatic ring containing one or more heteroatoms selected from N, O and S). Typical heteroaryl substituents include furyl, thienyl, pyrrole, pyrazole, triazole, thiazole, oxazole, pyridine, pyrimidine, isoxazolyl, pyrazine, indole, benzimidazole, and the like. When the heteroaryl ring is divalent it has been referred to as "heteroarylene" in this application.

It is understood that included in the family of compounds of Formula I as well as in Formulae Ia-Ij are isomeric forms including diastereoisomers, enantiomers, tautomers, and geometrical isomers in "E" or "Z" configurational isomer or a mixture of E and Z isomers. It is also understood that some isomeric forms such as diastereomers, enantiomers, and geometrical isomers can be separated by physical and/or chemical methods and by those skilled in the art.

Some of the inventive compounds may exist as single stereoisomers; racemates, and/or mixtures of enantiomers and /or diastereomers. All such single stereoisomers, racemates and mixtures thereof are intended to be within the scope of the subject matter described and claimed.

Additionally, Formula I is intended to cover, where applicable, solvated as well as unsolvated forms of the compounds. Thus, each formula includes compounds having the indicated structure, including the hydrated as well as the non-hydrated forms.

In addition to compounds of the Formula I, the HDAC inhibiting agents of the invention include pharmaceutically acceptable salts, prodrugs, and active metabolites of such compounds, and pharmaceutically acceptable salts of such metabolites.

Pharmaceutically acceptable salts include, when appropriate, pharmaceutically acceptable base addition salts and acid addition salts, for example, metal salts, such as alkali and alkaline earth metal salts, ammo-

nium salts, organic amine addition salts, and amino acid addition salts, and sulfonate salts. Acid addition salts include inorganic acid addition salts such as hydrochloride, sulfate and phosphate, and organic acid addition salts such as alkyl sulfonate, arylsulfonate, acetate, maleate, fumarate, tartrate, citrate and lactate. Examples of metal salts are alkali metal salts, such as lithium salt, sodium salt and potassium salt, alkaline earth metal salts such as magnesium salt and calcium salt, aluminum salt, and zinc salt. Examples of ammonium salts are ammonium salt and tetramethylammonium salt. Examples of organic amine addition salts are salts with morpholine and piperidine. Examples of amino acid addition salts are salts with glycine, phenylalanine, glutamic acid and lysine. Sulfonate salts include mesylate, tosylate and benzene sulfonic acid salts.

Preferred HDAC inhibiting agents include those having an IC_{50} value of 1 uM or less.

The hydroxamate compound, or salt thereof, is suitable for preparing pharmaceutical compositions, especially pharmaceutical compositions having deacetylase, especially histone deacetylase, inhibiting properties. The present subject matter disclosed herein further includes pharmaceutical compositions comprising a pharmaceutically effective amount of one or more of the above-described compounds as active ingredient. Pharmaceutical compositions according to the present disclosure are suitable for enteral, such as oral or rectal, and parenteral administration to mammals, including man, for the treatment of tumors, alone or in combination with one or more pharmaceutically acceptable carriers.

The hydroxamate compound is useful in the manufacture of pharmaceutical compositions having an effective amount the compound in conjunction or admixture with excipients or carriers suitable for either enteral or parenteral application. Preferred are tablets and gelatin capsules comprising the active ingredient together with (a) diluents; (b) lubricants, (c) binders (tablets); if desired, (d) disintegrants; and/or (e) absorbents, colorants, flavors and sweeteners. Injectable compositions are preferably aqueous isotonic solutions or suspensions, and suppositories are advantageously prepared from fatty emulsions or suspensions. The compositions may be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure and/or buffers. In addition, the compositions may also contain other therapeutically valuable substances. The compositions are prepared according to conventional mixing, granulating or coating methods, respectively, and contain preferably about 1 to 50% of the active ingredient.

Suitable formulations also include formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

As discussed above, the compounds of the embodiments disclosed are useful for treating proliferative

diseases. A proliferative disease is mainly a tumor disease (or cancer) (and/or any metastases). The inventive compounds are particularly useful for treating a tumor which is a breast cancer, genitourinary cancer, lung cancer, gastrointestinal cancer, epidermoid cancer, melanoma, ovarian cancer, pancreas cancer, neuroblastoma, head and/or neck cancer or bladder cancer, or in a broader sense renal, brain or gastric cancer; in particular (i) a breast tumor; an epidermoid tumor, such as an epidermoid head and/or neck tumor or a mouth tumor; a lung tumor, for example a small cell or non-small cell lung tumor; a gastrointestinal tumor, for example, a colorectal tumor; or a genitourinary tumor, for example, a prostate tumor (especially a hormone-refractory prostate tumor); or (ii) a proliferative disease that is refractory to the treatment with other chemotherapeutics; or (iii) a tumor that is refractory to treatment with other chemotherapeutics due to multidrug resistance.

In a broader sense of the embodiments set out herein, a proliferative disease may furthermore be a hyperproliferative condition such as leukemias, hyperplasias, fibrosis (especially pulmonary, but also other types of fibrosis, such as renal fibrosis), angiogenesis, psoriasis, atherosclerosis and smooth muscle proliferation in the blood vessels, such as stenosis or restenosis following angioplasty.

Where a tumor, a tumor disease, a carcinoma or a cancer are mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis.

The compound is selectively toxic or more toxic to rapidly propiferating cells than to normal cells, particularly in human cancer cells, e.g., cancerous tumors, the compound has significant antiproliferative effects and promotes differentiation, e.g., cell cycle arrest and apoptosis. In addition, the hydroxamate compound induces p21, cyclin-CDK interacting protein, which induces either apoptosis or G1 arrest in a variety of cell lines.

Additionally compounds of the various embodiments disclosed herein may be useful for treating neurodegenerative diseases, and inflammation.

SYNTHESIS OF DEACETYLASE INHIBITORS

The agents of the various embodiments may be prepared using the reaction routes and synthesis schemes as described below, employing the techniques available in the art using starting materials that are readily available. The preparation of particular embodiments is described in detail in the following examples, but the artisan will recognize that the chemical reactions described may be readily adapted to prepare a number of other agents of the various embodiments. For example, the synthesis of non-exemplified compounds may be successfully performed by modifications apparent to those skilled in the art, e.g., by appropriately protecting interfering groups, by changing to other suitable reagents known in the art, or by making routine modifications of reaction conditions. Alternatively, other reactions disclosed herein or known in the art will be recognized as having applicability for preparing other compounds of the various embodiments. Reagents useful for synthesizing compounds may be obtained or prepared according to techniques known in the art.

In the examples described below, unless otherwise indicated, all temperatures in the following description are in degrees Celsius and all parts and percentages are by weight, unless indicated otherwise.

Various starting materials and other reagents were purchased from commercial suppliers, such as Aldrich Chemical Company or Lancaster Synthesis Ltd., and used without further purification, unless otherwise indicated. Tetrahydrofuran (THF) and *N, N*-dimethylformamide (DMF) were purchased from Aldrich in SureSeal bottles and used as received. All solvents were purified by using standard methods in the art, unless otherwise indicated. TFA: trifluoroacetic acid.

The reactions set forth below were performed under a positive pressure of nitrogen, argon or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, and the reaction flasks are fitted with rubber septa for the introduction of substrates and reagents via syringe. Glassware was oven-dried and/or heat-dried. Analytical thin-layer chromatography was performed on glass-backed silica gel 60 F 254 plates (E Merck (0.25 mm)) and eluted with the appropriate solvent ratios (v/v). The reactions were assayed by TLC and terminated as judged by the consumption of starting material.

The TLC plates were visualized by UV absorption or with a *p*-anisaldehyde spray reagent or a phosphomolybdic acid reagent (Aldrich Chemical, 20wt% in ethanol) which was activated with heat, or by staining in iodine chamber. Work-ups were typically done by doubling the reaction volume with the reaction solvent or extraction solvent and then washing with the indicated aqueous solutions using 25% by volume of the extraction volume (unless otherwise indicated). Product solutions were dried over anhydrous sodium sulfate prior to filtration, and evaporation of the solvents was under reduced pressure on a rotary evaporator and noted as solvents removed in vacuo. Flash column chromatography [Still et al, *J. Org. Chem.*, 43, 2923 (1978)] was conducted using E Merck-grade flash silica gel (47-61 mm) and a silica gel:crude material ratio of about 20:1 to 50:1, unless otherwise stated. Hydrogenolysiss was done at the pressure indicated or at ambient pressure.

^{1}H NMR spectra were recorded on a Bruker AV400 instrument operating at 400 MHz, and ^{13}C-NMR spectra were recorded operating at 100 MHz. NMR spectra are obtained as $CDCl_3$ solutions (reported in ppm), using chloroform as the reference standard (7.26 ppm and 77.00 ppm) or CD_3OD (3.4 and 4.8 ppm and 49.3 ppm), or an internal tetramethylsilane standard (0.00 ppm) when appropriate. Other NMR solvents were used as needed. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets, tt = triplet of triplets. Coupling constants, when given, are reported in Hertz.

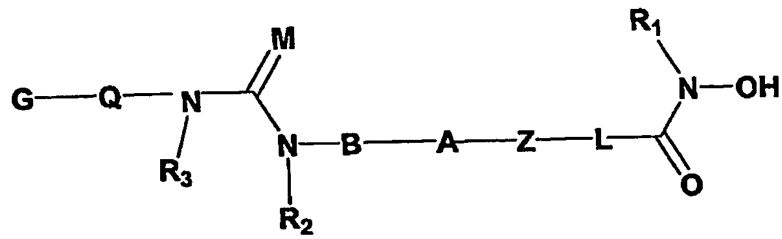
Mass spectra were obtained using LC-MS either in ESI or APCI. All melting points are uncorrected.

All final products had greater than 90% purity (by HPLC at wavelengths of 220 nm and 254 nm).

The following examples are intended to illustrate the embodiments disclosed and are not to be construed as being limitations thereto. Additional compounds, other than those described below, may be prepared using the following described reaction scheme or appropriate variations or modifications thereof.

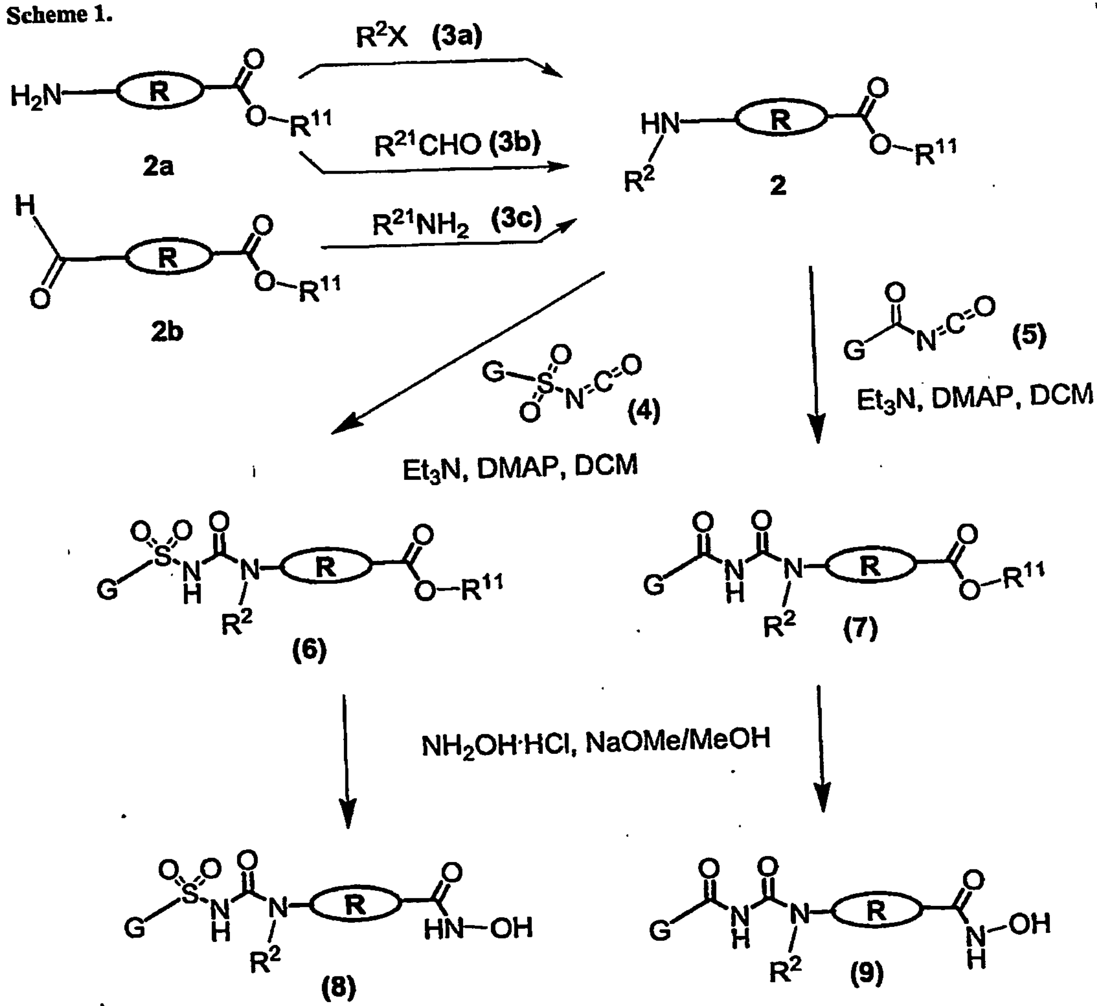
SYNTHESIS

Scheme 1 illustrates the procedure used for preparing compounds of formula I, wherein X and Y are hydrogens.



Formula (I)

Scheme 1.

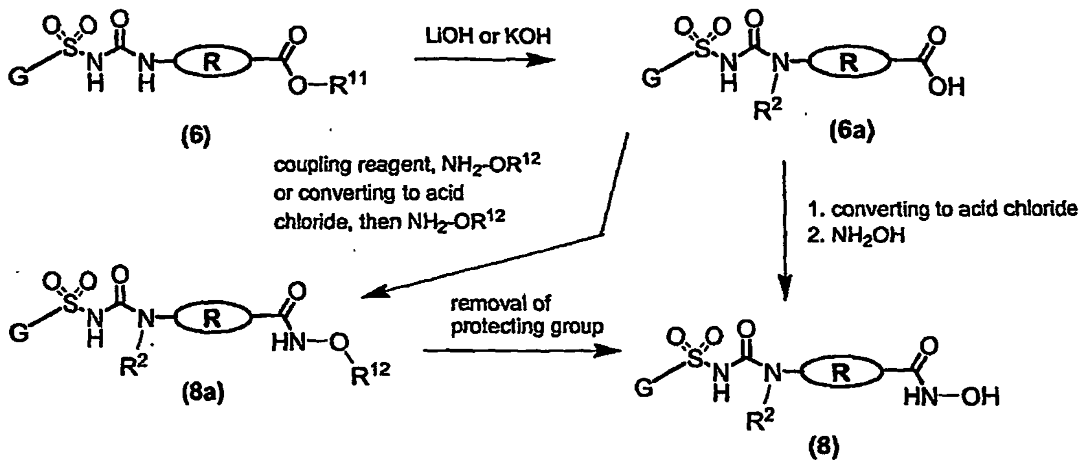


R is equal to –B-A-Z-L- as defined for formula I;

R^{21} is group R^2 less one CH_2.

The intermediate (2) could be prepared through by alkylation of amine (2a) with R^2X (3a, X is halo, e.g., I^-, Br^-, Cl^- or a good leaving group) or by reductive amination of aldehyde (2b) with amine $R^{21}NH_2$ (3b). Amine (2a) reacts with sulfonylisocyanate (4) to give sulfonylurea (6) which is converted to hydroxamic acid (8) by amination of the ester with hydroxylamine.

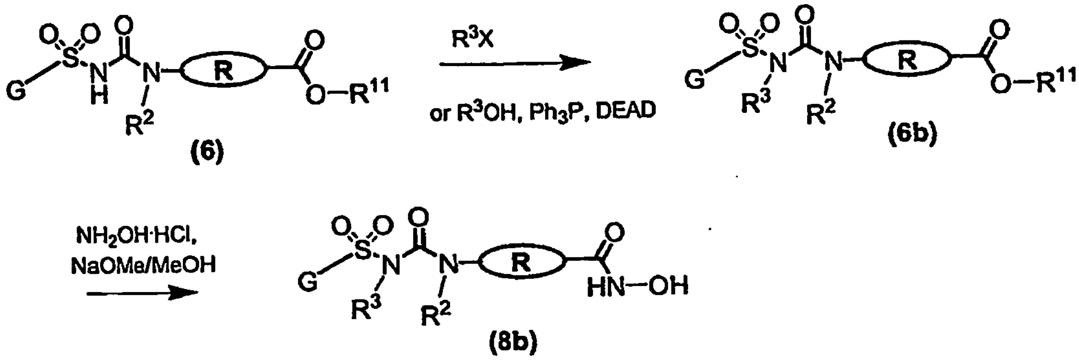
Scheme 2.



Other methods such as hydrolysis of the ester (6) to an acid, then (a): converting it to acid chloride by treating it with ClCOCOCl, or $SOCl_2$, or other methods under neutral conditions (Ph_3P with CBr_4, or 2,4,6-Trichloro-[1,3,5]triazine); or active ester by reacting it with isobutyl chloroformate; then reacting the acid chloride or active ester with hydroxylamine or the *O*-protected hydroxylamine (e.g., *O*-benzylhydroxylamie, *O*-(2,4-Dimethoxy-benzyl)-hydroxylamine, *O,N*-Bis-(2,4-dimethoxy-benzyl)-hydroxylamine, *O*-(Tetrahydro-pyran-2-yl)-hydroxylamine, *O*-(*tert*-Butyl-dimethyl-silyl)-hydroxylamine) to give the hydroxamic acid or the *O*-protected hydroxamic acids which need to remove the protecting group under the conditions known in the literatures or publication such as hydrogenolysis (to remove the benzyl) or acidic condition to cleave the acid labile protecting groups. (b) Coupling the acid with hydroxylamine or *O*-protected hydroxylamine with a coupling reagent.

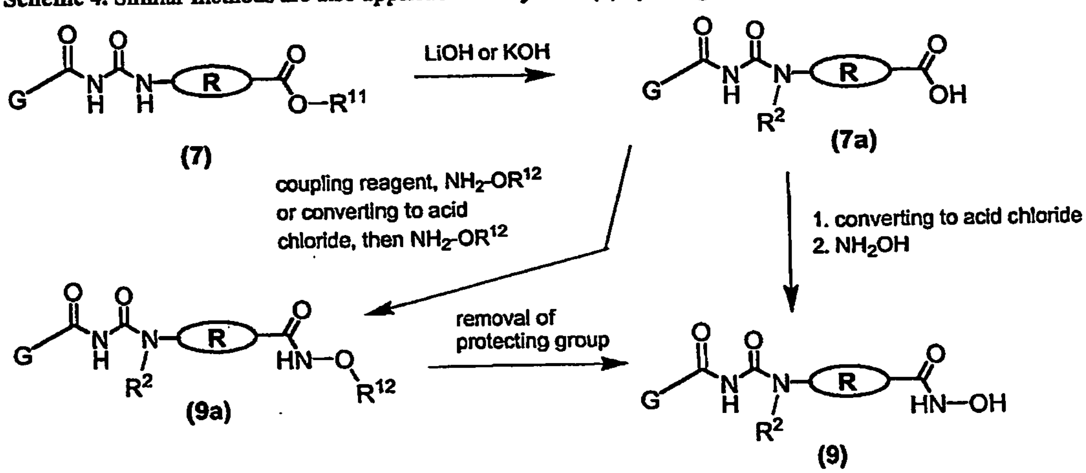
Scheme 3. Due to acidity of the sulfonylurea (6), the R^3 group could be introduced by alkylation of 6 with R^3X ($X = I^-$, Br^- or Cl^-) or by reacting with R^3OH under Mitsunobu reaction condition. The product (6b) could be converted to the hydroxamic acid (8b) by using the similar condition as for (8) in Scheme 1 and 2.

Scheme 3.

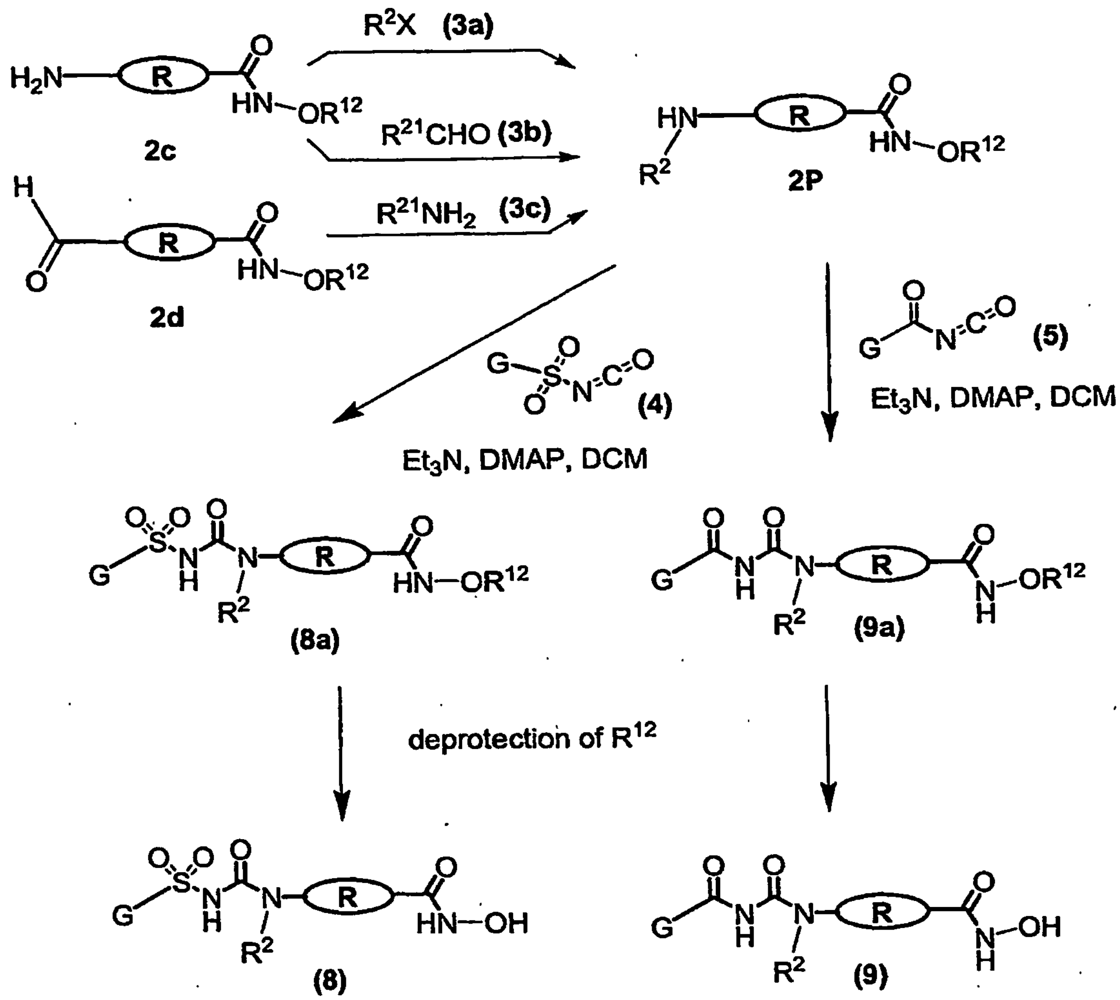


Scheme 1. By using the similar synthetic route, amine **(2a)** reacts with acylisocyanate **(5)** to give acylurea **(7)** which is converted to hydroxamic acid **(9)** by amination of the ester with hydroxylamine.

Scheme 4. Similar methods are also applicable to acylurea **(9)** by using *O*-protected hydroxylamine.



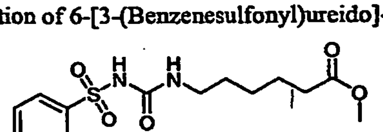
Scheme 5. Alternative methods for synthesis of sulfonylurea and acylurea: using O-protected hydroxamate starting material amine **(2c)** or aldehyde **(2d)** to make the *O*-protected hydroxamate intermediate **(2P)** which is converted to the corresponding sulfonylurea **(8a)** and acylurea **(9a)**. After removal the protecting group, sulfonylurea **(8)** and acylurea **(9)** could be obtained.



The following preparation and examples are given to enable those skilled in the art to more clearly understand and to practice the subject matter hereof. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

INTERMEDIATE 1

Preparation of 6-[3-(Benzenesulfonyl)ureido]-hexanoic acid methyl ester



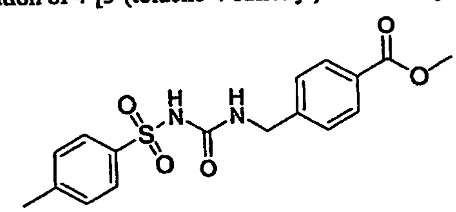
To a mixture of 6-Amino-hexanoic acid methyl ester hydrochloride (0. 10 g, 0.5 mmol), triethylamine (0.12 g, 1.2 mmol, 0.17 mL) and DMAP (0.06 g, 0.05 mmol) in the presence of CH_2Cl_2 (5 mL) was added phenyl sulfonyl isocyanate (0.12 g, 0.6 mmol). The reaction mixture was stirred at room temperature for 4 days. The reaction mixture was diluted with water (10 mL) and extracted with CH_2Cl_2 (3 x 10 mL). The combined organic extracts were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The crude

residue was chromatographed (silica) with 1-10% MeOH in CH_2Cl_2 to give 6-[3-(Benzenesulfonyl)ureido]-hexanoic acid methyl ester (0.1 g, 0.3 mmol, 58%) as a colorless oil which solidified on standing.

^{1}H NMR ($CDCl_3$) δ 7.95-7.89 (2H, m, aromatic protons), 7.66-7.53 (3H, m, aromatic protons), 3.67 (3H, s, CH_3), 3.22 (2H, q, J = 6.9 Hz, NHCH_2CH$_2$), 2.29 (2H, t, J = 7.4 Hz, CH_2COOMe), 1.62 (2 H, qn, J = 7.4 Hz), 1.50 (2 H, qn, J = 7.4 Hz) and 1.31-1.27 (2H, m).

INTERMEDIATE 2

Preparation of 4-[3-(toluene-4-sulfonyl)ureidomethyl-benzoic acid methyl ester

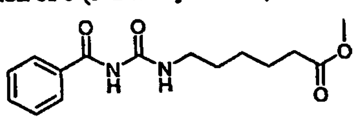


Proceeding as described in Intermediate 1 above but using appropriate starting materials. Yield: 67%. Light yellow solid. LC-MS (ESI, positive mode) m/z 363 ([M+H]$^+$).

^{1}H NMR (DMSO-d_6) δ 10.78 (bs, 1H), 7.85 (d, 2H, J = 8.2 Hz), 7.78 (d, 2H, J = 8.2 Hz), 7.40 (d, 2H, J = 8.0 Hz), 7.23 (d, 2H, J = 8.1 Hz), 4.22 (d, 2H, J = 5.9 Hz), 3.84 (s, 3H), 2.40 (s, 3H).

INTERMEDIATE 3

Preparation of 6-(3-Benzoyl-ureido)-hexanoic acid methyl ester



To a mixture of 6-Amino-hexanoic acid methyl ester hydrochloride (0. 05 g, 0.27 mmol), triethylamine (0.069 g, 0.6 mmol, 0.096 mL) and DMAP (0.03 g, 0.027 mmol) in the presence of CH_2Cl_2 (2 mL) was added benzoyl isocyanate (0.048 g, 0.3 mmol). The reaction mixture was stirred at room temperature for 4 days. The reaction mixture was diluted with water (10 mL) and extracted with CH_2Cl_2 (3 x 10 mL). The combined organic extracts were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The crude residue was chromatographed with 1 – 10% MeOH in CH_2Cl_2 to give 6-(3-Benzoyl-ureido)-hexanoic acid methyl ester (0.087 g, 0.2 mmol, quantitative yield) as a colorless oil which solidified on standing.

^{1}H NMR ($CDCl_3$) δ 8.66 (2 H, bs), 7.89-7.87 (2H, m, aromatic protons), 7.62-7.48 (3 H, m, aromatic protons), 3.67 (3 H, s, OCH_3), 3.39 (2 H, q, J = 7.0 Hz, NHCH_2CH$_2$), 2.33 (2H, t, J = 7.4 Hz, CH_2COOMe), 1.70-1.60 (4 H, m) and 1.45-1.40 (2 H, m).

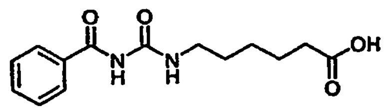
Large scale

To a mixture of 6-Amino-hexanoic acid methyl ester hydrochloride (0.363 g, 2.00 mmol), triethylamine (0.558 mL, 4.00 mmol) and DMAP (0.024 g, 0.20 mmol) in the presence of CH_2Cl_2 (10 mL) was added benzoyl isocyanate (0.276 mL, 2.20 mmol). The reaction mixture was stirred at room temperature for 6 h.

The reaction mixture was added brine and extracted with 10% methanol in dichloromethane. The extract was dried and concentrated and purified by reverse-phase HPLC to give 6-(3-Benzoyl-ureido)-hexanoic acid methyl ester (0.459 g, 79%).

INTERMEDIATE 4

Preparation of 6-(3-Benzoyl-ureido)-hexanoic acid

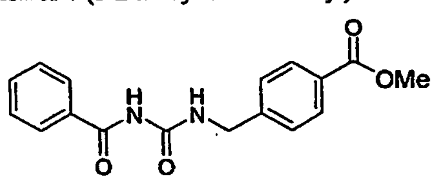


To a solution of 6-(3-Benzoyl-ureido)-hexanoic acid methyl ester (0.043 g, 0.14 mmol) in dry MeOH (2 mL) was added $NH_2OH.HCl$ (0.015 g, 0.2 mmol) followed by NaOMe (0.08 mL, 5.38M, 0.4 mmol). The reaction mixture was stirred at room temperature under nitrogen for 2 hours. The formation of the hydroxamic acid was followed by LCMS. Upon consumption of the starting material, the reaction mixture was diluted with acetonitrile and the solvent was removed *in vacuo*. The crude residue was purified by mass induced HTP. No hydroxamic acid was obtained but the corresponding 6-(3-Benzoyl-ureido)-hexanoic acid was obtained as a white fluffy solid.

^1H NMR (DMSO-d_6) δ 10.6 (1 H, s), 8.60 (1H, bs), 7.92-7.90 (2H, m, aromatic protons), 7.58-7.55 (1H, m, aromatic protons), 7.47-7.43 (2H, m, aromatic protons), 3.20-3.15 (2 H, m, $NHCH_2CH_2$), 2.16 (2 H, t, J = 7.3 Hz, CH_2COOMe), 1.52-1.42 (4 H, m) and 1.30-1.24 (2H, m).

INTERMEDIATE 5

Preparation of 4-(3-Benzoyl-ureidomethyl)-benzoic acid methyl ester



To a solution of 4-Aminomethyl-benzoic acid methyl ester hydrochloride (0.120 g, 0.60 mmol), triethylamine (0.125 mL, 0.90 mmol) and DMAP (0.006 g, 0.06 mmol) in CH_2Cl_2 (2 mL) was added benzoyl isocyanate (0.075 mL, 0.60 mmol). The reaction mixture was stirred at room temperature for 21 h and was extracted with ethyl acetate plus small amount of methanol.. The extract was dried ($MgSO_4$) and concentrated. The residue was purified by preparative reverse-phase HPLC. 4-(3-Benzoyl-ureidomethyl)-benzoic acid methyl ester was obtained as white solid (0.0486 g, 30%).

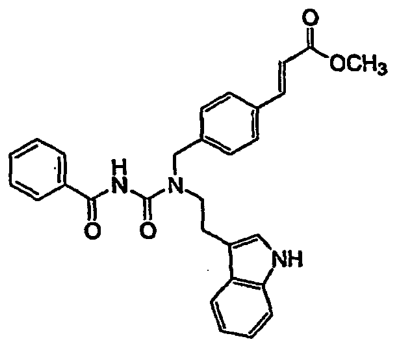
HPLC purity at 254nm: 95%;

LC-MS (ESI, positive mode) m/z 313 ($[M+H]^+$);

^1H NMR (DMSO-d_6) δ 9.12 (1H, t, J = 5.4 Hz), 8.68 (1H, s), 8.04 (2H, dt, J = 8.4, 1.8 Hz), 7.89 (2H, dt, J = 8.2, 1.6 Hz), 7.62 (1H, tt, J = 7.4, 1.8 Hz), 7.50 (2H, t, J = 8.0 Hz), 7.44 (2H, d, J = 8.4 Hz), 4.64 (2H, d, J = 6.0 Hz), 3.93 (3H, s).

INTERMEDIATE 6

Preparation of 3-(4-{3-Benzoyl-1-[2-(1H-indol-3-yl)-ethyl]-ureidomethyl}-phenyl)-acrylic acid methyl ester



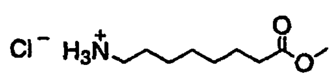
To a solution of 3-(4-{[2-(1H-Indol-3-yl)-ethylamino]-methyl}-phenyl)-acrylic acid methyl ester (0.100 g, 0.300 mmol), triethylamine (0.063 mL, 0.45 mmol) and DMAP (0.004 g, 0.03 mmol) in CH_2Cl_2 (3 mL) was added benzoyl isocyanate (90% pure, 0.045 mL, 0.36 mmol). The reaction mixture was stirred at room temperature for 22 h and was extracted with ethyl acetate. The extract was dried ($MgSO_4$) and concentrated. The residue was purified by HTP. 3-(4-{3-Benzoyl-1-[2-(1H-indol-3-yl)-ethyl]-ureidomethyl}-phenyl)-acrylic acid methyl ester was obtained as pale yellow solid (0.072 g, 50%).

LC-MS (ESI, positive mode) m/z 482 ($[M+H]^+$);

^1H NMR (DMSO-d_6) δ 10.73 (1H, s), 10.21 (1H, s), 7.75 (2H, d, J = 7.4 Hz), 7.66 (2H, d, J = 8.2 Hz), 7.59 (1h,d, J = 16.1 Hz), 7.53 (1H, t, J = 7.4 Hz), 7.43 (2H, t, J = 7.6 Hz), 7.36 (2H, broad d like), 7.29 (1H, m), 7.23 (1H, d, J = 8.1 Hz), 7.02 (1H, bs like), 6.94 (1H, t, J = 7.4 Hz), 6.58 (1H, d, J = 16.1 Hz), 4.61 (2H, s), 3.44 (2H, m), 2.89 (2H, m).

INTERMEDIATE 7

Preparation of 8-Amino-octanoic acid methyl ester hydrochloride



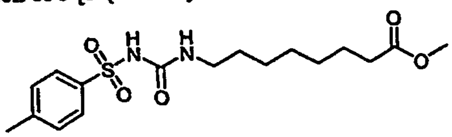
To a 100 mL round-bottomed flask, 8-Amino-octanoic acid (2.116 g, 13.29 mmol) and methanol (50 mL) were added. The mixture was stirred and cooled in a dry-ice /acetone bath under nitrogen. $SOCl_2$ (1.5 mL, 20.7 mmol) was added via syringe, then the dry-ice bath was removed and the mixture was stirred at room temperature for 2.5 h. The solution was evaporated and the residue was added diethyl ether. The solid was filtered and dried under vacuum. 8-Amino-octanoic acid methyl ester hydrochloride was obtained as white solid (2.772 g, 99.8%).

LC-MS (ESI, positive mode) m/z 376 ($[M-Cl]^+$).

^1H NMR (DMSO-d_6) δ 8.24 (3H, s, NH_3^+), 3.67 (3H, s, OCH_3), 3.00 (2H, m), 2.30 (2H, t, J = 7.5 Hz), 1.78 (2H, penta, J = 7.3 Hz), 1.61 (2H, penta, J = 7.2 Hz), 1.41 (2H, m), 1.39-1.32 (4H, m); ^{13}C NMR (DMSO-d_6) δ 174.1, 51.4, 39.9, 33.9, 28.6, 27.5, 26.3, 24.7.

INTERMEDIATE 8

Preparation of 8-[3-(4-methylbenzenesulfonyl)ureido]octanoic acid methyl ester



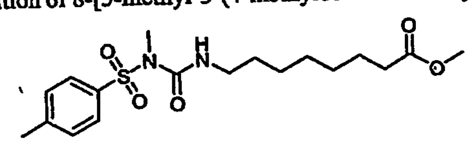
To a mixture of 8-Amino-octanoic acid methyl ester hydrochloride (0.601 g, 2.865 mmol), triethylamine (1.0 mL, 7.18 mmol) and DMAP (0.0313 g, 0.256 mmol) in CH_2Cl_2 (20 mL) was added *p*-toluene sulfonylisocyanate (0.63 mL, 4.12 mmol). The reaction mixture was stirred at room temperature for 19.5 h. The reaction mixture was diluted with 1N HCl and extracted with CH_2Cl_2 (100 mL x1, 50 mL x 2). The combined organic extracts were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The crude residue was chromatographed (silica) with 2–10% MeOH in CH_2Cl_2 to give 8-[3-(4-methylbenzenesulfonyl)ureido]octanoic acid methyl ester (0.730 g, 69%) as a white solid.

LC-MS (ESI, positive mode) m/z 371 ($[M+H]^+$).

^1H NMR (CDCl$_3$) δ 8.80 (1H, bs), 7.78 (2H, d, J = 8.3 Hz), 7.31 (2H, d, J = 8.1 Hz), 6.52 (1H, t, J = 5.2 Hz), 3.67 (3H, s, OCH_3), 3.19 (2H, q, J = 6.6 Hz), 2.44 (3H, s, Ar-CH_3), 2.30 (2H, t, J = 7.5 Hz), 1.63-.1.58 (2H, m), 1.48-1.42 (2H, m), 1.31-1.22 (6H, m); ^{13}C NMR (DMSO-d_6) δ 174.3, 151.9, 144.6, 136.8, 129.6, 127.0, 51.5, 40.2,34.0, 29.4, 28.9, 28.8, 26.6, 24.8, 21.6

INTERMEDIATE 9

Preparation of 8-[3-methyl-3-(4-methylbenzenesulfonyl)ureido]octanoic acid methyl ester



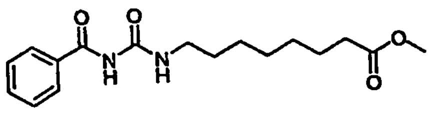
To a mixture of 8-[3-(4-methylbenzenesulfonyl)ureido]octanoic acid methyl ester (0.161 g, 0.435 mmol), K_2CO_3 (0.572 g, 4.14 mmol) and acetonitrile (4 mL) was added MeI (0.270 mL, 4.35 mmol). The reaction mixture was stirred at room temperature under nitrogen for 17 h. The reaction mixture was diluted with 1N HCl and extracted with ethyl acetate ($Na_2S_2O_3$ was added to the aqueous layer to reduce the I_2). The combined organic extracts were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. ^1H NMR of the crude residue (0.166 g) showed that the molar ratio of 8-[3-(4-methylbenzenesulfonyl)ureido]octanoic acid methyl ester to degraded product 4,*N*,*N*-Trimethyl-benzenesulfonamide was 3:1.

LC-MS (ESI, positive mode) m/z 385 ($[M+H]^+$).

^1H NMR (CDCl$_3$) δ 7.70 (2H, d, J = 8.3 Hz), 7.34 (2H, d, J = 8.5 Hz), 3.66 (3H, s, OCH_3), 3.24 (2H, q, J = 5.7 Hz), 3.12 (3H, s, NCH_3), 2.43 (3H, s, Ar-CH_3), 2.31 (2H, t, J = 7.5 Hz), 1.65-1.60 (2H, m), 1.55-1.51 (2H, m), 1.33-1.26 (6H, m).

INTERMEDIATE 10

Preparation of 8-(3-Benzoyl-ureido)-octanoic acid methyl ester



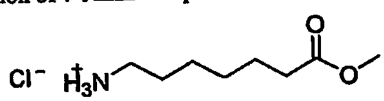
To a solution of 8-Amino-octanoic acid methyl ester hydrochloride (0.423 g, 2.02 mmol), triethylamine (0.56 mL, 4.02 mmol) and DMAP (0.022 g, 0.18 mmol) in CH_2Cl_2 (10 mL) was added benzoyl isocyanate (90% pure, 0.370 g, 2.26 mmol). The reaction mixture was stirred at room temperature for 2 h and was added silica gel and filtered through silica and washed with ethyl acetate. The filtrate was evaporated to dryness to give colorless oil (0.691 g, 106%), which was solidified at room temperature under vacuum.

LC-MS (ESI, positive mode) m/z 321 ($[M+H]^+$).

^{1}H NMR ($CDCl_3$) δ 10.52 (1H, s), 8.92 (1H, t, J = 5.6 Hz), 8.09 (2H, d, J = 7.2 Hz), 7.56 (1H, t like), 7.45 (2H, t, J = 7.7 Hz), 3.64 (3H, s, OCH_3), 3.35 (2H, q, J = 6.0 Hz), 2.29 (2H, t, J = 7.5 Hz), 1.63-1.56 (4H, m), 1.39-1.32 (6H, m); ^{13}C NMR ($CDCl_3$) δ 173.6, 168.1, 154.6, 132.3, 132.0, 128.0, 127.7, 50.9, 39.3, 33.5, 29.0, 28.5, 28.4, 26.3, 24.3.

INTERMEDIATE 11

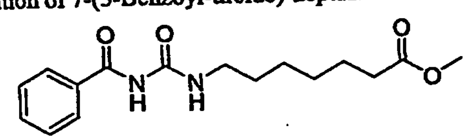
Preparation of 7-Amino-heptanoic acid methyl ester hydrochloride



Proceeding as described in Intermediate 7 above but using appropriate starting materials (7-Amino-heptanoic acid), the titled compound was prepared as white solid (0.490 g, 100%).

INTERMEDIATE 12

Preparation of 7-(3-Benzoyl-ureido)-heptanoic acid methyl ester



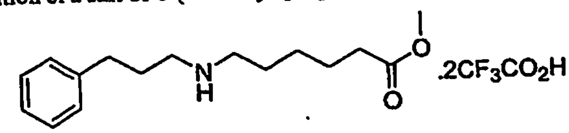
Proceeding as described in Intermediate 10 above but using appropriate starting materials (7-Amino-heptanoic acid methyl ester hydrochloride), the crude titled compound was obtained as oil which was solidified under vacuum and could be used for next step of reaction without further purification.

LC-MS (ESI, positive mode) m/z 307 ($[M+H]^+$).

^{1}H NMR ($CDCl_3$) δ 10.30 (1H, s), 8.90 (1H, t, J = 5.4 Hz), 8.05 (2H, d, J = 7.5 Hz), 7.57 (1H, t, J = 7.4 Hz), 7.47 (2H, d, J = 7.6 Hz), 3.65 (3H, s, OCH_3), 3.35 (2H, q, J = 6.6 Hz), 2.30 (2H, t, J = 7.5 Hz), 1.68-1.56 (4H, m), 1.45-1.35 (4H, m); ^{13}C NMR ($CDCl_3$) δ 173.6, 168.1, 154.5, 132.4, 132.0, 128.1, 127.6, 51.0, 39.3, 33.5, 28.9, 28.3, 26.1, 24.3.

INTERMEDIATE 13

Preparation of a salt of 6-(3-Phenyl-propylamino)-hexanoic acid methyl ester. 2 TFA



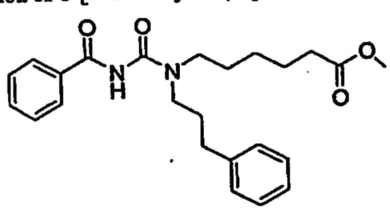
To a 100 mL flask, 6-Amino-hexanoic acid methyl ester hydrochloride (0.555 g, 3.06 mmol), $NaBH(OAc)_3$ (0.782 g, 3,.69 mmol), 3-Phenyl-propionaldehyde (0.47 mL, 3.21 mmol), dichloromethane (10 mL) and triethylamine (0.43 mL, 3.09 mmol) were added. The above mixture was sonicated for 1 min then stirred at room temperature overnight. The reaction mixture was added aqueous Na_2CO_3 and extracted with dichloromethane (x 2). The extract was dried and purified by reverse-phase preparative HPLC to give the titled compound as an oil (0.202 g, 30% calculated as a salt of 2TFA).

LC-MS (ESI, positive mode) m/z 264 ($[M+H]^+$).

^{1}H NMR (CDCl$_3$) δ 11.82 (1H, s), 8.62 (2H, s, -NH_2^+-), 7.26 (2H, t, J = 7.3 Hz), 7.18 (1H, t, J = 7.3 Hz), 7.11 (2H, d, J = 7.0 Hz), 3.64 (3H, s, OCH_3), 2.94 and 2.92 (each of 2H, overlapped, identified by COSY), 2.63 (2H, t, J = 7.5 Hz), 2.26 (2H, t, J = 7.3 Hz), 2.00 (2H, penta, J = 7.6 Hz), 1.65 (2H, penta, J = 7.5 Hz), 1.55 (2H, penta, J = 7.7 Hz), 1.33 (2H, m); ^{13}C NMR (CDCl$_3$) δ 173.6, 139.2, 128.2, 127.7, 126.0, 51.1, 47.3, 47.1, 32.9, 32.0, 26.9, 25.2, 25.0, 23.4.

INTERMEDIATE 14

Preparation of 6-[3-Benzoyl-1-(3-phenyl-propyl)-ureido]-hexanoic acid methyl ester



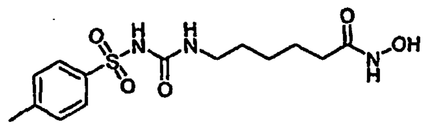
Proceeding as described in Intermediate 10 above but using appropriate starting materials (a salt of 6-(3-Phenyl-propylamino)-hexanoic acid methyl ester with 2 TFA), the crude titled compound was purified by reverse-phase preparative HPLC and flash chromatography (silica, 5% Methanol in dichloromethane) to give the pure compound as gum (0.063 g, 43%).

LC-MS (ESI, positive mode) m/z 411 ($[M+H]^+$).

^{1}H NMR (CDCl$_3$) δ 8.20 (1H, bs), 7.78 (2H, bs), 7.54 (1H, t, J = 7.4 Hz), 7.43 (2H, t, J = 7.6 Hz), 7.24 (2H, d, J = 7.2 Hz), 7.18-7.13 (3H, m), 3.65 (3H, s), 3.36 [4H, m or 3.38 (2H, m) and 3.36 (2H, m)], 2.64 (2H, t, J = 7.4 Hz), 2.30 (2H, t, J = 7.4 Hz), 1.96 (2H, penta, J = 7.4 Hz), 1.66-1.56 (4H, m), 1.31 (2H, m); ^{13}C NMR (CDCl$_3$) δ 173.5, 165.8 (br), 153.4 (br), 140.7 (br), 132.7, 132.1, 128.1, 128.0, 127.9, 127.3, 125.6, 51.0, 46.9 (br, 2 x CH_2N), 33.4, 32.4, 28.9, 27.1 (br), 25.8, 24.1.

EXAMPLE 1

Preparation of 6-[3-(toluene-4-sulfonyl)ureido]-hexanoic acid hydroxyamide

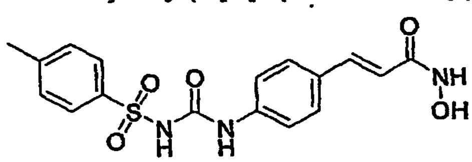


To a solution of 6-[3-(toluene-4-sulfonyl)ureido]-hexanoic acid methyl ester (0.035 g, 0.1 mmol) in dry MeOH (2 mL) was added $NH_2OH.HCl$ (0.021 g, 0.3 mmol) followed by NaOMe (0.11 mL, 5.38M, 0.6 mmol). The reaction mixture was stirred at room temperature under nitrogen for 2 hours. The formation of the hydroxamic acid was followed by LCMS. Upon consumption of the starting material, the reaction mixture was diluted with acetonitrile and the solvent was removed *in vacuo*. The crude residue was purified by mass induced HPLC purification system to give 6-[3-(toluene-4-sulfonyl)ureido]-hexanoic acid hydroxyamide as a pale yellow/whitish solid.

^{1}H NMR (DMSO-d_6) δ 10.37 (1H, bs), 10.13 (1H, s), 8.46 (1 H, s), 7.60 (2H, d, J = 8.3 Hz, aromatic CH), 7.23 (2H, d, J = 8.0 Hz, CH), 6.27 (1H, t, J = 5.2 Hz), 2.92 (2H, q, J = 6.1 Hz), 2.39 (3H,s), 1.89 (2H, t, J = 7.5 Hz), 1.43 (2H, penta, J = 7.5 Hz), 1.31 (2H, penta, J = 7.4 Hz), 1.17-1.09 (2H, m).

EXAMPLE 2

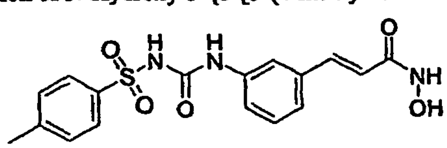
Preparation of *N*-Hydroxy-{3-[4-[3-(toluene-4-sulfonyl)ureido]-phenyl}-acrylamide



Yield: 5% from the corresponding methyl ester. White solid. HPLC purity at 254nm: 93%;
LC-MS (ESI, positive mode) m/z 376 ([M+H]$^+$).

EXAMPLE 3

Preparation of N-Hydroxy-3-{3-[3-(4-methylbenzenesulfonyl)ureido]-phenyl}-acrylamide

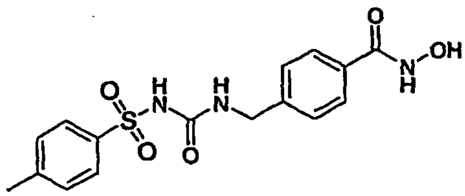


Prepared from the corresponding methyl ester. Yield: 64%. White solid. HPLC purity at 254nm: 95%;
LC-MS (ESI, positive mode) m/z 376 ([M+H]$^+$);

^{1}H NMR (DMSO-d_6) δ 7.70 (d, 2H, J = 6.0 Hz), 7.36 (d, 2H, J = 8.1 Hz), 7.30 (d, 1H, J = 15.8 Hz), 7.25 (s, 1H), 7.11 (t, 1H, J = 7.7 Hz), 6.80 (d, 1H, J = 8.2 Hz), 6.68 (d, 1H, J = 7.6 Hz), 6.33 (d, 1H, J = 15.8 Hz), 2.37 (s, 3H, -CH_3).

EXAMPLE 4

Preparation of 4-[3-(toluene-4-sulfonyl)ureidomethyl-*N*-hydroxy-benzamide

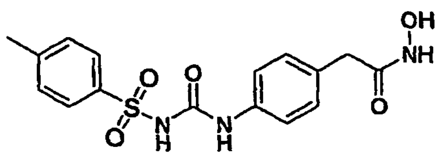


Prepared from the corresponding methyl ester. Yield: 58%. White solid. HPLC purity at 254nm: 100%; LC-MS (ESI, positive mode) m/z 364 ($[M+H]^+$);

^{1}H NMR (DMSO-d_6) δ 11.14 (s, 1H), 10.74 (s, 1H), 8.98 (d, 1H, J = 1.7 Hz), 7.79 (d, 2H, J = 8.3), 7.65 (d, 2H, J = 8.3 Hz), 7.41 (d, 2H, J = 8.0 Hz), 7.18 (d, 2H, J = 8.2 Hz), 7.05 (t, 1H, J = 5.8 Hz), 4.19 (d, 2H, J = 5.9), 2.47 (s, 3H).

EXAMPLE 5

Preparation of methyl-*N*-hydroxy-benzamide *N*-Hydroxy-2-{4-[3-(toluene-4-sulfonyl)ureido]-phenyl}-acetamide

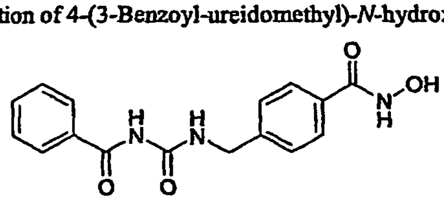


Prepared from the corresponding methyl ester. Yield: 99%. White solid. HPLC purity at 254nm: 99%. LC-MS (ESI, positive mode) m/z 364 ($[M+H]^+$);

^{1}H NMR (DMSO-d_6) δ 10.47 (s, 1H), 8.64 (s, 1H), 7.73 (d, 2H, J = 8.2 Hz), 7.32 (d, 2H, J = 8.1 Hz), 7.13 (d, 2H, J = 8.5 Hz), 7.02 (d, 2H, J = 8.3 Hz), 3.08 (s, 2H), 2.29 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 167.0, 149.2, 141.9, 137.1, 130.8, 129.4, 129.2, 127.4, 125.6, 118.8, 38.6, 21.0.

EXAMPLE 6

Preparation of 4-(3-Benzoyl-ureidomethyl)-*N*-hydroxy-benzamide

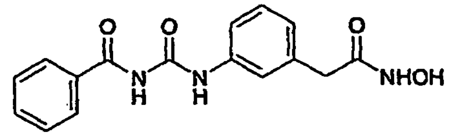


To a solution of 4-(3-Benzoyl-ureidomethyl)-benzoic acid methyl ester (0.030 g, 0.096 mmol) in dry MeOH (0.5 mL) was added $NH_2OH.HCl$ (0.020 g, 0.288 mmol) followed by 30% NaOMe solution (5.38 M, 0.106 mL, 0.576 mmol). The reaction mixture was stirred at room temperature under nitrogen for 22 hours then quenched by addition of concentrated hydrochloric acid. The mixture was subjected to preparative HPLC for purification. A white solid obtained (0.035 g, purity 64%) which was re-purified by preparative HPLC to give 4-(3-Benzoyl-ureidomethyl)-*N*-hydroxy-benzamide (yield 47%).

HPLC purity at 254nm: 99.7%, t_R = 4.55 min.
LC-MS (ESI, positive mode) m/z 314 ($[M+H]^+$);
^{1}H NMR (DMSO-d_6) δ 11.11 (s, 1H), 10.71 (s, 1H), 9.04-9.07 (tr, 1H, J = 6.0 Hz), 8.92 (br s, 1H), 7.89-7.91 (d, 2H, J = 8.4 Hz), 7.65-7.67 (d, 2H, J = 8.3 Hz), 7.54-7.58 (m, 1H), 7.42-7.48 (m, 2H), 7.32-7.34 (d, 2H, J = 8.3 Hz), 4.42-4.43 (d, 2H, J = 6.0 Hz), 2.47 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 167.5, 153.0, 141.8, 132.0, 131.8, 130.7, 128.4, 127.8, 127.7, 127.4, 126.3, 41.8.

EXAMPLE 7

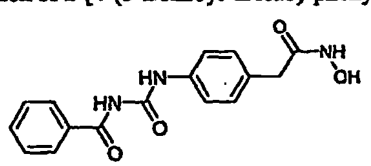
Preparation of 2-[3-(3-Benzoyl-ureido)-phenyl]-*N*-hydroxy-acetamide



Prepared from the corresponding methyl ester. Yield: 7%. White solid. HPLC purity at 254nm: 99%;
LC-MS (ESI, positive mode) m/z 314 ($[M+H]^+$);
^{1}H NMR (DMSO-d_6) δ 11.02 (s, 1H), 10.84 (s, 1H), 10.66 (s, 1H), 8.83 (s, 1H), 8.02 (d, 2H, J = 8.5 Hz), 7.65 (m, 1H), 7.53-7.57 (m, 2H), 7.50 (dd, 1H, J = 8.1 Hz), 7.44 (s, 1H), 7.27-7.30 (t, 1H, J = 7.8 Hz), 7.01 (d, 1H, J = 7.8 Hz), 3.29 (s, 2H).

EXAMPLE 8

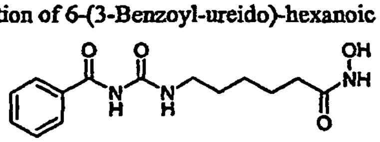
Preparation of 2-[4-(3-Benzoyl-ureido)-phenyl]-*N*-hydroxy-acetamide



Prepared from the corresponding methyl ester. Yield: 2%. White solid. HPLC purity at 254nm: 98%;
LC-MS (ESI, positive mode) m/z 314 ($[M+H]^+$).

EXAMPLE 9

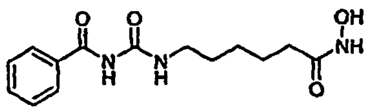
Preparation of 6-(3-Benzoyl-ureido)-hexanoic acid hydroxyamide



To a solution of 6-(3-Benzoyl-ureido)-hexanoic acid (0.0033 g, 0.01 mmol) in DMF (1 mL) was added Py-BOP (0.07 g, 0.013 mmol) and Hunig's base (0.013 mL, 0.07 mmol). The reaction mixture was stirred for 5 minutes and the $NH_2OH.HCl$ (0.02 g, 0.02 mmol) was added. The reaction mixture was stirred overnight at room temperature under nitrogen. The crude reaction mixture was purified by mass induced HPLC to give 6-(3-Benzoyl-ureido)-hexanoic acid hydroxyamide as an off white solid

EXAMPLE 10

Preparation of 6-(3-Benzoyl-ureido)-hexanoic acid hydroxyamide



To a solution of 6-(3-Benzoyl-ureido)-hexanoic acid methyl ester (0.300 g, 1.03 mmol) in dry MeOH (2.0 mL) was added $NH_2OH.HCl$ (0.555 g, 8.00 mmol) followed by 30% NaOMe in MeOH (2.23 mL, 5.38M, 12.0 mmol). The reaction mixture was stirred at room temperature under nitrogen for 1 h then was added trifluoroacetic acid (0.3 mL) in an ice-bath. The solution was extracted with 10% MeOH in dichloromethane. The extract was dried and concentrated. The residue was purified by reverse-phase preparative HPLC to give 6-(3-Benzoyl-ureido)-hexanoic acid hydroxyamide (0.175 g, 59%) as white solid.

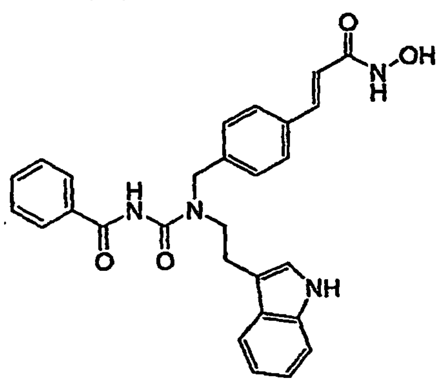
LC-MS (ESI, positive mode) m/z 293 ($[M+H]^+$);

HPLC purity at 254 nm: 99.7%, t_R = 5.15 min.

^1H NMR (DMSO-d_6) δ 10.63 (1H, s), 10.34 (1H, s), 8.70-8.60 (1H, bs), 8.65 (1H, t, J = 5.7 Hz, $CONHCH_2$), 7.95 (2H, dt, J = 7.2, 1.6 Hz), 7.62 (1H, tt, J = 7.4, 1.2 Hz), 7.50 (2H, t, J = 7.9 Hz), 3.22 (2H, q, J = 6.6 Hz, CH_2N), 1.96 (2H, t, J = 7.4 Hz, CH_2CO), 1.56-1.46 (4H, m), .130-1.24 (2H, m); ^{13}C NMR (DMSO-d_6) δ 169.1 (CONHOH), 168.2 (PhCO), 153.4 (NHCONH), 132.7 (CH), 132.6 (Cq), 128.4 (CH x 2), 128.0 (CH x 2), 38.9 (CH_2N), 32.2 (CH_2CO), 28.9, 25.9, 24.8.

EXAMPLE 11

Preparation of 3-(4-{3-Benzoyl-1-[2-(1H-indol-3-yl)-ethyl]-ureidomethyl}-phenyl)-N-hydroxy-acrylamide

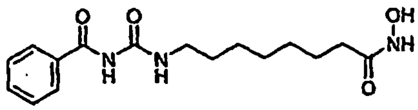


To a solution of 3-(4-{3-Benzoyl-1-[2-(1H-indol-3-yl)-ethyl]-ureidomethyl}-phenyl)-acrylic acid methyl ester (0.030 g, 0.062 mmol) in dry MeOH (0.3 mL) was added $NH_2OH.HCl$ (0.033 g, 0.48 mmol) followed by 30% NaOMe in MeOH (0.134 mL, 5.38M, 0.72 mmol). The reaction mixture was stirred at room temperature under nitrogen for 3 h then was neutralized with trifluoroacetic acid. The mixture was purified by reverse-phase HPLC to give 3-(4-{3-Benzoyl-1-[2-(1H-indol-3-yl)-ethyl]-ureidomethyl}-phenyl)-N-hydroxy-acrylamide (0.0037 g, 12%) as pale yellow solid.

LC-MS (ESI, positive mode) m/z 483 ($[M+H]^+$).

EXAMPLE 12

Preparation of 8-(3-Benzoyl-ureido)-octanoic acid hydroxyamide.



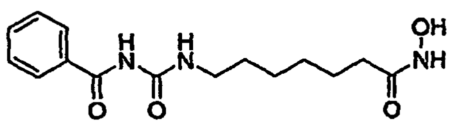
To a solution of 8-(3-Benzoyl-ureido)-octanoic acid methyl ester (0.275 g, equal to 0.811 mmol) and $NH_2OH.HCl$ (0.562 g, 8.09 mmol) was added dry MeOH (5 mL) and followed by NaOMe in MeOH (2.30 mL, 4.37 M, 10.0 mmol). The reaction mixture was stirred at room temperature under nitrogen for 50 min then was neutralized with trifluoroacetic acid (0.80 mL). The mixture was purified by reverse-phase preparative HPLC (C_{18}, 5 um, 21.2x150 mm, 20 mL/min, 5 to 95% of CH_3CN + 0.05% TFA over 18 min), to give 8-(3-Benzoyl-ureido)-octanoic acid hydroxyamide as white powder (0.115 g, 44%).

LC-MS (ESI, positive mode) m/z 322 ($[M+H]^+$).

^{1}H NMR (DMSO-d_6) δ 10.64 (1H, s), 10.34 (1H, s), 8.70-8.60 (1H, bs), 8.66 (1H, t, J = 5.1 Hz, $CONHCH_2$), 7.96 (2H, d, J = 7.5 Hz), 7.62 (1H, t, J = 7.0 Hz), 7.50 (2H, t, J = 7.3 Hz), 3.23 (2H, q, J = 6.1 Hz, CH_2N), 1.95 (2H, t, J = 7.2 Hz, CH_2CO), 1.50-1.48 (4H, m), 1.29-1.22 (6H, m); ^{13}C NMR (DMSO-d_6) δ 169.1 (CONHOH), 168.2 (PhCO), 153.5 (NHCONH), 132.7 (CH), 132.6 (Cq), 128.4 (CH x 2), 128.1 (CH x 2), 39.0 (CH_2N), 32.2 (CH_2CO), 29.1, 28.5, 28.4, 26.3, 25.0.

EXAMPLE 13

Preparation of 7-(3-Benzoyl-ureido)-heptanoic acid hydroxyamide.



Proceeding as described in Example 12 above but using appropriate starting materials (7-(3-Benzoyl-ureido)-heptanoic acid methyl ester), and the reaction mixture was neutralized by TFA and evaporated to dryness. The residue was washed with water and the titled compound was obtained as white solid (0.175 g, 67% in two steps).

LC-MS (ESI, positive mode) m/z 308 ($[M+H]^+$).

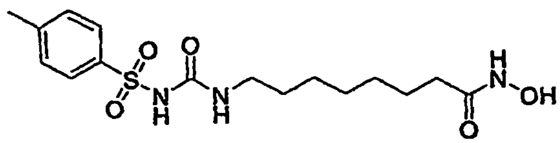
HPLC purity at 254 nm: 98.7%

^{1}H NMR (DMSO-d_6) δ 10.64 (1H, s), 10.35 (1H, s), 8.70-8.60 (1H, bs), 8.64 (1H, t, J = 5.6 Hz, $CONHCH_2$), 7.96 (2H, d, J = 7.4 Hz), 7.62 (1H, t, J = 7.4 Hz), 7.50 (2H, t, J = 7.7 Hz), 3.22 (2H, q, J = 6.5 Hz, CH_2N), 1.95 (2H, t, J = 7.3 Hz, CH_2CO), 1.55-1.40 (4H, m), 1.35-1.20 (4H, m); ^{13}C NMR (DMSO-d_6) δ 169.1 (CONHOH), 168.2 (PhCO), 153.4 (NHCONH), 132.7 (CH), 132.6 (Cq), 128.4 (CH x 2), 128.1 (CH x 2), 39.0 (CH_2N), 32.2, 29.1, 28.2, 26.1, 25.0.

The following compounds are prepared by method analogous to those disclosed in Example 1:

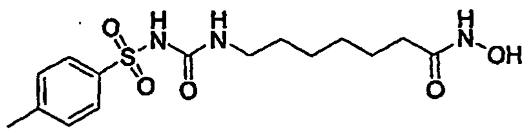
EXAMPLE 14

8-[3-(4-methylbenzenesulfonyl)-ureido])-octanoic acid hydroxyamide:



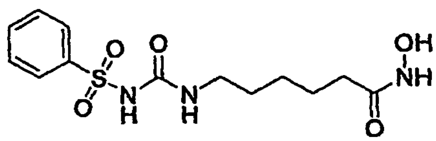
EXAMPLE 15

7-[3-(4-methylbenzenesulfonyl)-ureido])-heptanoic acid hydroxyamide:



EXAMPLE 16

6-[3-(benzenesulfonyl)-ureido])-hexanoic acid hydroxyamide



BIOLOGICAL TESTING AND ENZYME ASSAYS

Recombinant GST-HDAC1 Protein expression and purification

Human cDNA library was prepared using cultured SW620 cells. Human HDAC1 coding region was amplified from this cDNA library, and cloned into pDEST20 vector using GATEWAY Cloning Technology (Invitrogen). The pDEST20-HDAC1 construct was confirmed by DNA sequencing. pDEST20-HDAC1 was then transformed into *Escherichia coli* DH10Bac competent cells. pDEST20-HDAC1 bacmid was isolated from the positive white colony. Recombinant baculovirus was prepared using the Bac-To-Bac method following the manufacturer's instruction (Invitrogen). The steps involved transfecting SF9 cells with the isolated bacmid using CELLFECTIN reagent (Invitrogen), followed by two-round of virus amplifications. Baculovirus titer was determined by plaque assay to be about 10^8 PFU/ml.

Expression of GST-HDAC1 was done by infecting SF9 cells with pDEST20-HDAC1 baculovirus at MOI=1 for 60 h. Cells were harvested by centrifugation at 500g for 10 min at 4°C. The cell pellets were suspended in lysis buffer (4ml/g of pellet) containing 50mM HEPES, pH7.5, 250mM NaCl, 1% Triton X-100, 3mM $MgCl_2$, 1mM DTT, 1mM PMSF, 20μg/ml DNaseI and 1x Protease Inhibitor cocktail (Roche). Cell lysate was then subjected to 3 freeze/thaw cycles in liquid N_2/37°C water bath. Cell debris was removed from the soluble cell lysate by centrifugation at 13,200rpm for 30 min at 4°C. Soluble cell lysate was then incubated with pre-equilibrated Glutathione Sepharose 4B beads (Amersham) at 4°C for 2 h. Unbound protein was removed from the mixture by a centrifugation at 500g for 5 min at 4°C. The beads were

washed with PBS buffer for 3 times. And GST-HDAC1 protein was eluted by elution buffer containing 50mM Tris, pH8.0, 150mM NaCl, 1% Triton X-100 and 10mM or 20mM reduced Glutathione. Purified GST-HDAC1 protein was dialyzed with HDAC storage buffer containing 10mM Tris, pH7.5, 100mM NaCl and 3mM $MgCl_2$. 20% Glycerol was added to purified GST-HDAC1 protein before storage at -80°C.

Equipments:

(a). Baculovirus culture incubator and shaking incubator, set at 27.5°C (Sanyo, Labnet, IKA and Infors, Swiss)

(b). Biological Safety cabinet (Gelman and NuAire)

Materials:

(a). SF9 cells (Invitrogen)

(b). SF-900 II SFM medium (Invitrogen)

(c). Disposable polycarbonate Erlenmeyer flasks (Corning)

(d). CELLFECTIN reagent (Invitrogen)

(e). 6-well tissue culture dishes (Falcon)

(f). Concert High Purity Plasmid Miniprep (Marligen)

(g). Glutathione Sepharose 4B beads (Amersham)

In vitro HDAC assay for determination of IC50 values

Experimental procedure

The assay has been carried out in 96well format and the BIOMOL fluorescent-based HDAC activity assay has been applied. Compared to the traditional HDAC assay-using radioisotope labeled substrate [1, 11, 19], this assay is more specific (p53 peptide substrate for HDAC8 [2, 5]), easier (two steps), homogenous and sensitive (fluorescent-based). Briefly, deacetylation of the substrate sensitizes it to the developer, which then generates a fluorophore (symbol). Te fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader. Currently, this assay has been successfully applied in many studies related to HDAC inhibition effects [4, 12, 14, 21]. The analytical software, Prism 3.0 has been used to generate IC50 from a series of data. The pipetting scheme for a representative experiment is shown below: 1. add 10 ul of assay buffer into columns 2-5, 7-10, wells B,C,D,E,G11-12 ; 2. add 12.5 ul of 2.5X compound (2.5 uM) into columns1, 6 and wells A11-12. 12.5 ul buffer into wells F11 and12; 3. serials dilute 2.5 ul (5X) in orientation as indicated above; 4. discard last 2.5 ul from column5, 10 and wells E11-12; 5. add 2.5 ul of HDAC enzyme (0.5 U for HDAC8, 2 ul for HDAC1 to reach final concentration of 600 nM) into all wells except F11-12; 6. add 12.5 ul of 2X substrate (200 uM for peptide substrate, 500 uM for generic substrate) into all wells; 7. incubate at RT for 2 hr with agitation; 8. add 25 ul of 2X developer into all wells and incubate for 10 mins.

Equipment & Materials

Equipment:

(a). Tecan Ultra Microplate detection system (Tecan Group Ltd. Switzerland)

(b). Labnet Shaker, Model 30 (National Labnet Co., Inc. Woodbridge, NJ, USA)

Materials:

(a). 96-well U-form black microplate, 650209, (Greiner Bio-One, Frickenhausen, Germany)

(b). Histone Deacetylase 8 (HDAC8) (human, recombinant), 100 U (SE-145, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(c). HeLa nuclear extract, 100 ul (KI-140, BIOMOL Research Laboratories, Inc. Plymouth Meeting. PA, USA)

(d). *Fluor de Lys*-HDAC8 Substrate, 0.5 umol (KI-178, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(e). *Fluor de Lys*- substrate, 0.5 umol (KI-104, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(f). *Fluor de Lys*™ Developer Concentrate (20x), 300 ul (KI-105, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(g). HDAC assay buffer: Tris pH7.5, 25 mM; NaCl, 137 mM; KCl, 2.7 mM; $MgCl_2$, 1 mM, BSA,

Data analysis

The assay is composed of duplicates for each compound. Thus, for the raw values from fluorescence reading, a mean value will be calculated using Excel formula [average (value1: value2)]; in addition, standard deviation will also be determined based on duplicates by formula [std (value1: value2)]. The Z' factor is calculated on the basis of high/ low signal with definitions: high signal= no inhibitor and low signal= no enzyme. Hence $Z'=1-3*(SD_{high}+SD_{low})/(Signal_{high}-Signal_{low})$.

The HDAC enzyme inhibition results of representative compounds are shown in Table 2 & 3.

Table 2

Compound	HDAC8 Enzyme Activity, IC_{50} (μM)
Example 1	0.282
Example 3	0.022
Example 6	0.075
Example 10	0.561
Example 11	0.124
Example 14	0.051
SAHA	0.234

Table 3

Compound	HDAC1 Enzyme Activity, IC_{50} (μM)
Example 1	>100
Example 3	5.66
Example 6	0.38
Example 10	0.064
Example 11	0.002
Example 14	1.13
SAHA	0.100

Cell-based proliferation assay for determination of GI50 values

The Cell proliferation assay is performed in a 96-well plate format. Cells are plated overnight and treated with compounds (in triplicates, 9-dose treatment, 4-fold dilutions from 100uM) over 96 hrs. Cell growth is then determined by analyzing the number of viable cells remaining following treatment of the cells. Dose response curves are plotted to determine GI_{50} values for the compounds. Staurosporine treatment is used as a positive control for the experiments as staurosporine inhibits kinases and has anti-proliferative activity.

The CyQUANT cell proliferation assay is a fluorescent assay based on the measurement of cellular nucleic acid content. It contains a fluorescent nucleic acid stain, the CyQUANT GR reagent, that measures total nucleic acids as a direct indication of cell number. The assay is more rapid and convenient than conventional assays measuring metabolic activity as it does not require long incubations and cells can be frozen and stored prior to assaying. In this protocol, it is applied solely to the analysis of adherent cell lines. After treatment with compounds, detached dead cells are removed with the culture supernatant and only the viable cells remaining are quantified in the assay by fluorescence measurement at 485/535nm. In the analysis of viable cells in suspension cell lines, the CyQuant assay cannot distinguish between live and dead cells in suspension as it measures total nucleic acid content (from both live and dead cells) in solution. Thus, for analysis of suspension cell lines, methods based on measurement of metabolic activity have to be used.

The method adapted for this protocol is the Celltiter96 Aq_{ueous} One Solution Cell Proliferation Assay. It is a colorimetric method for determination of cell viability based on the cleavage of an MTS tetrazolium compound into a colored formazan product by metabolically active cells. The quantity of formazan product is directly proportional to the number of living cells in culture. The assay has to be performed immediately after compound treatment of cells. The Celltiter96 Aq_{ueous} One Solution Reagent is added directly to the

cells in culture and incubated for 1-4hours for color development before recording the absorbance at 490nm.

Equipment & Materials

Equipment:

Vortex Mixer

Plate shaker for 96-well plates

Single and Multichannel pipettors

Cell culture Incubator

Microplate readers for reading of fluorescence(485/535nm) and absorbance (490nm)

Materials:

Appropriate cancer cell line(s) and culture media

10mM Compounds

1mM Staurosporine

96-well cell culture plates

Reagent reservoirs

For adherent cell lines: CyQUANT cell proliferation assay kit (Molecular Probes #C-7026)

For suspension cell lines: Celltiter96 Aq_{ueous} One Solution Cell Proliferation Assay (Promega #G3580)

The cell activity results of representative compounds are shown in Table 4.

Table 4

Compound	NCI H552 (μM)	Colon 205 (μM)
Example 6	2.00	0.69
Example 10	0.50	2.02
Example 11	4.62	0.38
SAHA	1.29	2.57

Histone H3 acetylation assay

A hallmark of histone deacetylase (HDAC) inhibition is the increase in the acetylation level of histones. The degree of histone acetylation can be monitored by a Western Blot approach, where specific antibodies directed against the acetylated version of histone H3 are used. Briefly, 1.5 x10^6 Colo 205 colon cancer cells were plated into 10 cm dishes and grown overnight in RPMI medium. Thereafter, the cells were treated with increasing amounts of HDAC inhibitory compounds by adding them into the medium (0.1, 1, 5 and 10 μM final concentration). After 12 hours of incubation the cells were harvested, lysates prepared and the Western Blot procedure carried out as described in detail below.

Western Blot approach

Specific proteins can readily be identified with the use of antibodies directed explicitly towards it. Accordingly, the more abundant protein would display a stronger signal compared to one which was present in a lower concentration.

Proteins must first be extracted from cells and quantified before equal amounts from each cell line can be separated by gel electrophoresis (SDS-PAGE).

Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for Western Blot analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane (nitrocellulose or PVDF) in the same sequence of separation as that on the SDS-PAGE. The membrane is then blocked with an inert protein like bovine serum albumin (BSA) or non-fat milk. This will avoid non-specific binding of the primary antibody to the un-blotted surface of the membrane.

To detect the antigen (separated protein of interest) blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane.

In order to detect the antibody which has bound, an anti-immunoglobulin antibody coupled to a reporter group such as the enzyme horse radish peroxidase is added (e.g. Goat anti-human IgG- alkaline phosphatase). This anti-Ig-enzyme is commonly called a "second antibody" or "conjugate". Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody is bound to the protein.

PROTEIN EXTRACTION

Keep equipment cold and everything else on ice!!

PROTEIN EXTRACTION BUFFER (1 ml.): Take 200ul each of 5x Buffer, 5x NaCl, and 5x Igepal from the Sigma Mammalian Cell Lysis Kit, and make up to volume with 400ul of deionised water. Add 60 ul Protease Inhibitor Cocktail for every ml. of extraction buffer.(*Reduce Protease Inhibitor is higher volumes are used in protein extraction ~ 15ul for every ml)

Protein Extraction from cells grown in culture:

Rinse cells in ice-cold PBS and detach with trypsin. Pellet cells by centrifugation ~1300g x 5 minutes, and remove supernatant. Resuspend cell pellet in I ml. PBS and transfer into a 1.5ml. Eppendorf tube and centrifuge ~ 13000g for 10 mins. Remove supernatant and gently resuspend cells in appropriate amounts of PROTEIN EXTRACTION BUFFER (e.g. ~ 50ul for small pellets and 100 – 150ul for larger pellets) Immediately freeze in LN_2 and allow to thaw on ice ~ 20 mins. Centrifuge at ~13000 x g for 30 minutes Remove supernatant into a new tube and either freeze in LN_2 and store at -80° C or keep on ice for further work.

PROTEIN QUANTIFICATION (BRADFORD METHOD)

1. Prepare protein standards of 0 – 3000 ng from a stock solution of 250ug/ml; 2. dilute all samples between 50x – 100x; 3. dilute Bio-Rad Protein Assay solution 5x and transfer 200ul into each well in a 96-well plate; 4. add 20ul of each standard or sample solution into individual wells; 5. mix for ~ 1 min.; 6. read absorbance at 595 nm.

PROTEIN SEPARATION (SDS-PAGE)

6X Sample Loading Buffer

7 ml. 0.5M Tris-HCl, 0.4% SDS, 3 ml. glycerol, 1 g SDS, 0.93 g DTT, 1.2 mg bromophenol blue. Make up volume to 10 ml with deionised water. Store in small aliquots at –20C. Add an appropriate amount of sample loading buffer to all samples. Heat to 95° C in the heating block for 5 minutes.

GEL ELECTROPHORESIS:

Set up the Xcell Sure Lock Electrophoresis gel tank according to the manufacturer's instructions. Place the pre-cast NuPage gels into the gel holders and add diluted NuPage tank buffer into the chambers.

Load the Rainbow molecular weight marker and samples into respective wells in the gel

Connect the gel tank to the power pac and run the at 150V constant for ~ 1 hour.

PROTEIN TRANSFER – ELECTROBLOTTING

While gel is running cut PVDF membrane and filter papers to exact size of gel (8.5 x 6cm). Wet PVDF with methanol in petri-dish. Then soak PVDF in diluted NuPage transfer buffer for at least 15min. Take gel, dessemble and cut wells and stacking gel off. Make a gel sandwich (From black side down on bench):

-Sponge (make sure no bubbles)

-3x Whatman filter paper (Wet in transfer buffer)

-Gel, roll out bubbles

-PVDF, roll out bubbles

-3x Whatman filter paper (Wet in transfer buffer)

-Sponge (make sure no bubbles)

Assemble transfer apparatus. Place a small stirrer in chamber. Load transfer cassettes with the BLACK side FACING BLACK part of blotting chamber. Fill chamber with Transfer Buffer past all holes of sand-wich pack. Run at 100V for 60mins or 250mA for 150 mins at 4oC. Dissemble gel sandwich.

Mark the marker on the blot according to the color and identify the protein side up.

IMMUNODETECTION (ANTIBODIES)

Reagents:

10X Tris Saline Solution (TSS) (pH 7.6)

Tris 100 mM	12.1g/L
NaCl 1.5M	87.6g/L

Adjust pH to 7.6 and store at 0-4C

Blocking Solution (1x TSS/5% milk)

10x TSS	10ml
Non-fat powder milk	5g
Deionized water	90ml

Washing Buffer (1x TSS/0.5% milk /0.1% Tween-20)

10X TSS	100ml

Non-fat powder milk	2.5g
Tween-20	1ml
Deionized water	890ml

Blocking

Transferred proteins can be visualized by staining the membrane for a few minutes with Ponceau S. Remove stain from the membrane by washing with deionized water. Place membrane into blocking solution. Block for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C.

Incubation with primary antibody

Antibodies used:

I Anti-acetylated Histone H3 (Lysin 14), UPSTATE

II Anti-Actin (SIGMA)

Protocol

Decant the blocking buffer and wash the membrane with washing buffer for another 30mins.
Add the primary antibody, diluted in washing buffer as suggested in the product description sheet.
Incubate with agitation for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C.
Incubation with secondary antibody. Decant the primary antibody. Wash the membrane 3X with washing buffer. First, for 15 mins then the subsequent 2X for 5 minutes. Decant the wash solution and add HRP-conjugated secondary antibody, diluted in wash buffer. Incubate for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C. Decant the antibody conjugate and wash for 40 minutes with agitation in wash buffer, changing the wash buffer every 10 minutes.

Substrate incubation (ECL)

Decant washing buffer and place the blot on a clean tray.Prepare enough detection substrate according to the manufacturer's specifications. Gently drop the substrate solution over the protein side of the membrane and incubate at RT between 1-5 mins. Remove the blot from the tray and place it between two pieces of write-on transparency film. Smooth over the covered blot to remove air bubbles and excess substrate and place the blot in an X-ray cassette. In the dark room lay x-ray film down over blots for 1sec - 20mins. Remove and develop film in the X-ray film processor. *All work must be carried out in the dark or only with red light.*

Reseal the cassette and replace all films into the respective box before switching on the lights.

Data analysis

Place the developed film into the UVP under white light and use the Bioimaging software to read the density of each band observed on the film.

The values are then normalized against the density of actin (or any other house-keeping protein) in the corresponding samples to obtain the expression of the protein in a particular cell line.

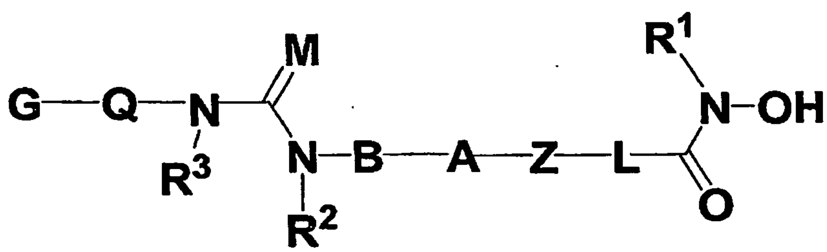
The results of histone deacetylase assay are shown in Table 5.

Table 5

Compound	Histone 3 acetylation activity
Example 6	Active
Example 10	Active
Example 11	Active
SAHA	Active

What is claimed is:

1. A compound of the formula (I)



Formula (I)

wherein

R^1 is selected from H, C_1 -C_6 alkyl, acyl;

L is a single bond or is a C_1-C_5 hydrocarbon chain which may contain 0 to 2 double bond or triple bond or one double bond and one triple bond, the chain may be optionally interrupted by -O-, -S-, -S(O)- and -$S(O)_2$-, and unsubstituted or substituted with one or more substituents independently selected from the group consisting of C_1-C_4 alkyl;

Z is single bond or selected from O, S, S(O), $S(O)_2$, etc.

A is single bond or a ring which is selected from arylene, heteroarylene, cycloalkylene and heterocycloalkylene which could be further optionally substituted by X and Y.

B is single bond or a ring which is selected from arylene, heteroarylene, arylalkylene, heteroarylalkylene, C_1-C_3 alkylene, heteroalkylene, cycloalkylene and heterocycloalkylene which could be further optionally substituted by X and Y.

M is selected from O, S, NH, NR^4, NOH and NOR^4.

R^2 and R^3 are the same or different and independently H, C_1 -C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 -C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), hydroxyl, hydroxyal-

kyl, alkoxy, amino, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR^4, -CONHR^4, -NHCONHR^4, C(=NOH)R^4, and acyl;

Q is selected from -S(O)$_2$-, -CO- and –C(=S)-.

G is selected from aryl, heteroaryl, alkyl, cycloalkyl, heterocycloalkyl, arylalkyl and heteroarylalkyl; they also could be further substituted by X, Y, R^4, hydroxyl, hydroxyalkyl, alkoxy, amino, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR^4, -C(O)OH, -SH, -CONHR^4, -NHCONHR^4, and C(=NOH)R^4.

X and Y are the same or different and independently selected from hydrido, halo, C_1 -C_4 alkyl, such as CH_3 and CF_3, NO_2, OR^4, SR^4, C(O)R^5, CN, and NR^6 R^7 ;

R^4 is selected from C_1-C_4 alkyl, heteroalkyl, aryl, heteroaryl, acyl;

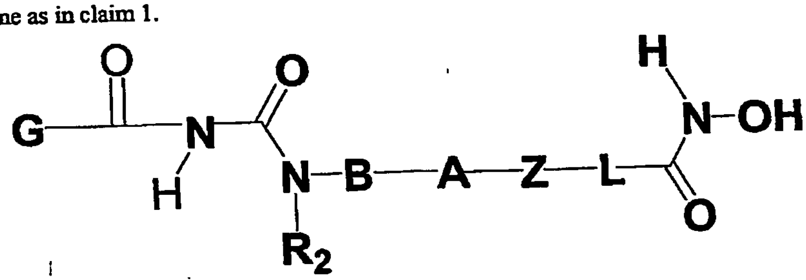
R_5 is selected from C_1-C_4 alkyl;

R^6 and R^7 are the same or different and independently selected from hydrido, C_1-C_6 alkyl, C_4-C_9 cycloalkyl, C_4-C_9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

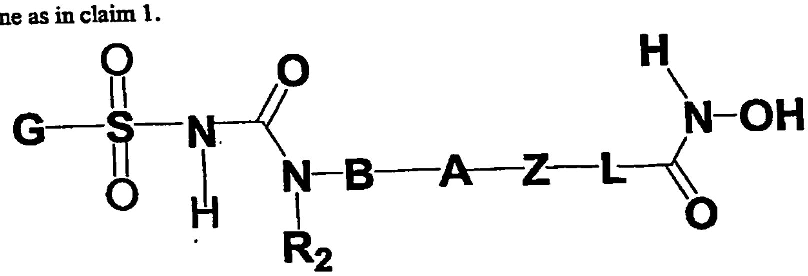
2. The compound according to claim 1 wherein each of R^1, R^3, X and Y is H.

3. The compound according to claim 1 of formula (Ia) wherein R^1 = R^3 = H; R^2, G, X, Y, Z, A, B, R^3 and R^4 are the same as in claim 1.



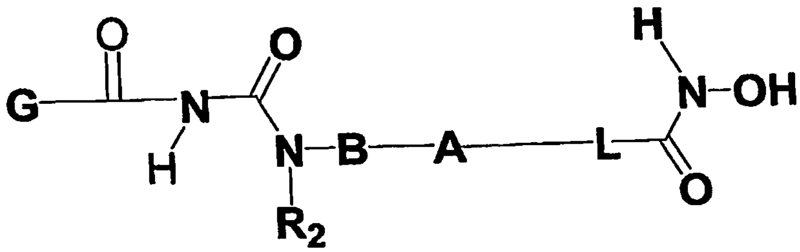
Formula (Ia)

4. The compound according to claim 1 of formula (Ib) wherein R^1 = R^3 = H; R^2, G, X, Y, Z, A, B, R^3 and R^4 are the same as in claim 1.



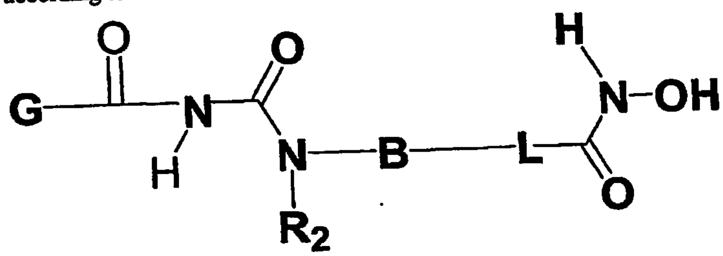
Formula (Ib)

5. The compound according to claim 3 of formula (Ic).



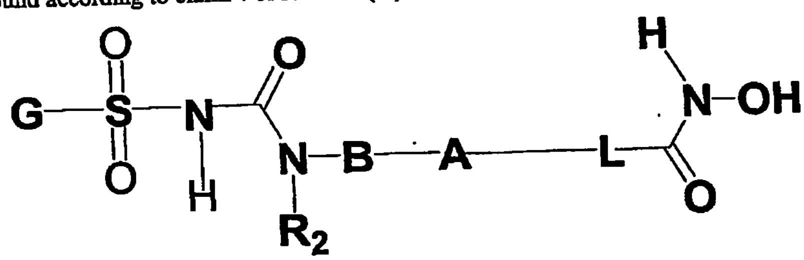
Formula (Ic)

6. The compound according to claim 3 of formula (Id).



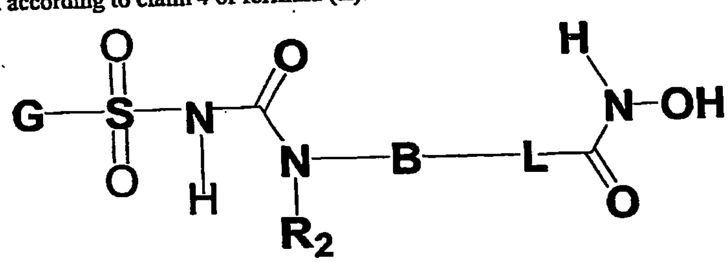
Formula (1d)

7. The compound according to claim 4 of formula (Ie)



Formula (Ie)

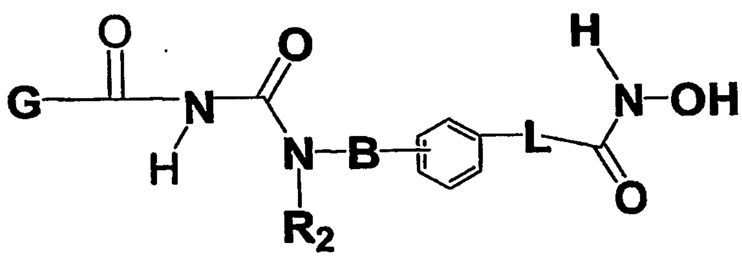
8. The compound according to claim 4 of formula (If).



Formula (If)

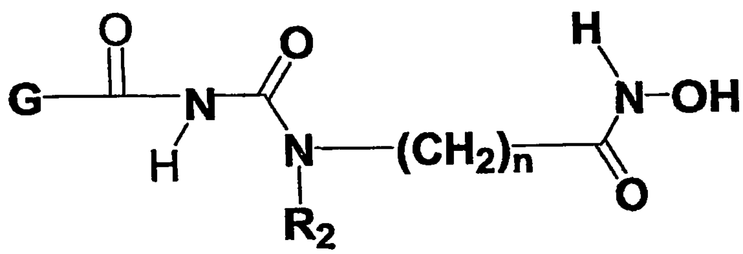
9. The compound according to claim 5 of formula (Ig) wherein B is a single bond or CH_2, L is a single bond or selected from CH_2, CH_2CH_2, –CH=CH-, -C-triple bond-C-, R^2 is selected from H, alkyl, arylalkyl,

arylheteroalkyl, heteroarylalkyl, heteroarylheteroalkyl. B is attached to meta or para position of phenylene against L. G is selected from aryl, heteroaryl, alkyl and alkoxyalkyl.



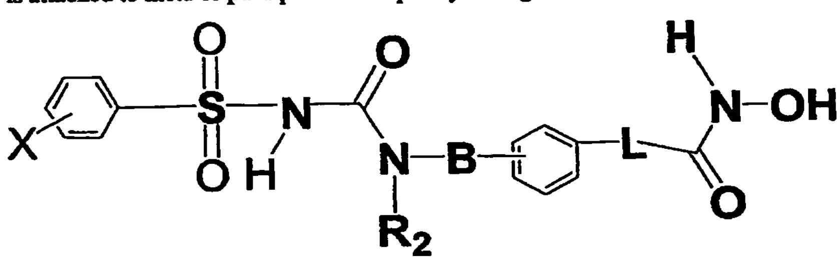
Formula (Ig)

10. The compound according to claim 6 of formula (Ih) wherein n is an integer from 1 to 8; R^2 is selected from H, alkyl, arylalkyl, arylheteroalkyl, heteroarylalkyl,. heteroarylheteroalkyl; G is selected from aryl, heteroaryl, alkyl and heteroalkyl.



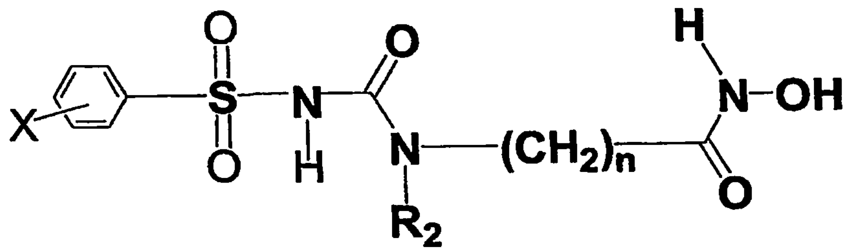
Formula (Ih)

11. The compound according to claim 7 of formula (Ii) wherein B is a single bond or CH_2, L is a single bond or selected from CH_2, CH_2CH_2, –CH=CH-, -C-triple bond-C-, R_2 is selected from H, alkyl, arylalkyl, arylheteroalkyl, heteroarylalkyl, heteroarylheteroalkyl. X is selected from H, halo, C_1-C_4 alkyl, alkxoy, alkylamino; B is attached to meta or para position of phenylene against L.



Formula (Ii)

12. The compound according to claim 8 of formula (Ij) wherein n is an integer from 1 to 8, X is selected from H, halo, C_1-C_4 alkyl, alkxoy, alkylamino.



Formula (Ij)

13. The use of a pharmaceutical composition comprising the compound according to claim 1 to treat proliferative diseases, including cancerous tumors.

14. The use of a pharmaceutical composition comprising the compound according to claim 3 to treat proliferative diseases, including cancerous tumors.

15. The use of a pharmaceutical composition comprising the compound according to claim 4 to treat proliferative diseases, including cancerous tumors.

16. The use of a pharmaceutical composition comprising the compound according to claim 9 to treat proliferative diseases, including cancerous tumors.

17. The use of a pharmaceutical composition comprising the compound according to claim 10 to treat proliferative diseases, including cancerous tumors.

18. The use of a pharmaceutical composition comprising the compound according to claim 1 to modify deacetylase activity, preferably histone deacetylase activity.

19. The use of a pharmaceutical composition comprising the compound according to claim 3 to modify deacetylase activity, preferably histone deacetylase activity.

20. The use of a pharmaceutical composition comprising the compound according to claim 4 to modify deacetylase activity, preferably histone deacetylase activity.

21. The use of a pharmaceutical composition comprising the compound according to claim 9 to modify deacetylase activity, preferably histone deacetylase activity.

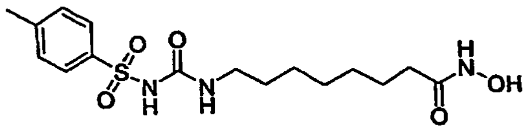
22. The use of a pharmaceutical composition comprising the compound according to claim 10 to modify deacetylase activity, preferably histone deacetylase activity.

23. The use of a pharmaceutical composition comprising the compound according to claim 4 to modify histone deacetylase activity, preferably HDAC8 activity.

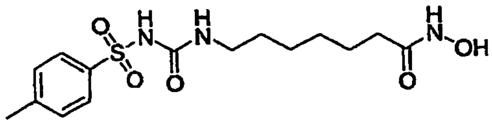
24. The use of a pharmaceutical composition comprising the compound according to claim 9 to modify histone deacetylase activity, preferably HDAC1 and HDAC8 activity.

25. The use of a pharmaceutical composition comprising the compound according to claim 10 to modify histone deacetylase activity, preferably HDAC1 and HDAC8 activity.

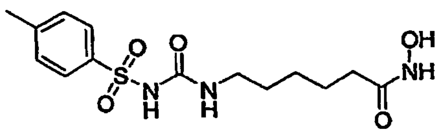
26. The use according to claims 13-25 wherein said compound is selected from compounds, and their pharmaceutically acceptable salts, selected from the group of compounds consisting of



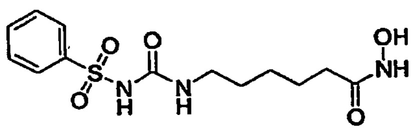
8-[3-(4-methylbenzenesulfonyl)-ureido])-octanoic acid hydroxyamide



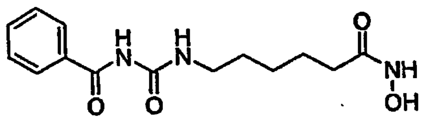
7-[3-(4-methylbenzenesulfonyl)-ureido])-heptanoic acid hydroxyamide



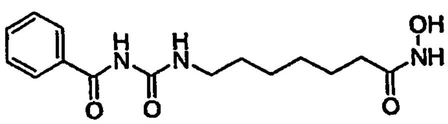
6-[3-(4-methylbenzenesulfonyl)-ureido])-hexanoic acid hydroxyamide



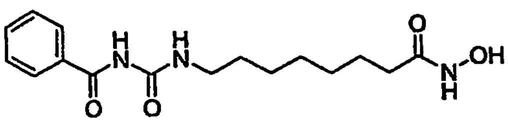
6-[3-(benzenesulfonyl)-ureido])-hexanoic acid hydroxyamide



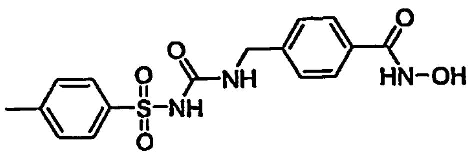
6-(3-Benzoyl-ureido)-hexanoic acid hydroxyamide



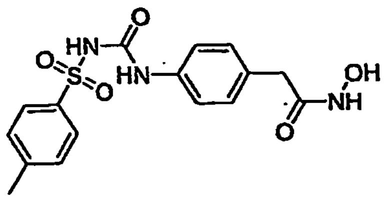
7-(3-Benzoyl-ureido)-heptanoic acid hydroxyamide



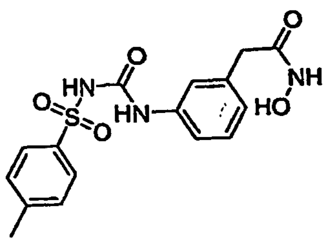
8-(3-Benzoyl-ureido)-octanoic acid hydroxyamide



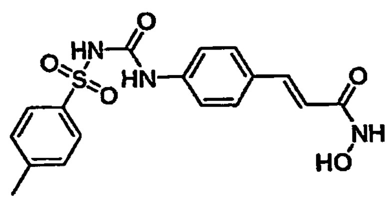
N-Hydroxy-4-[3-(4-methylbenzenesulfonyl)ureido]methyl-benzamide



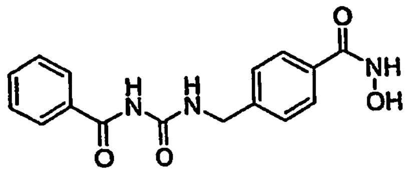
N-Hydroxy-2-{4-[3-(4-methylbenzenesulfonyl)ureido]-phenyl}-acetamide



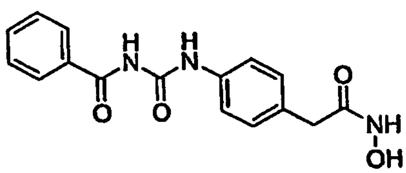
N-Hydroxy-2-{3-[3-(4-methylbenzenesulfonyl)ureido]-phenyl}-acetamide



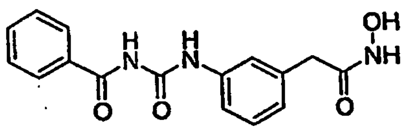
N-Hydroxy-3-{4-[3-(4-methylbenzenesulfonyl)ureido]-phenyl}-acrylamide



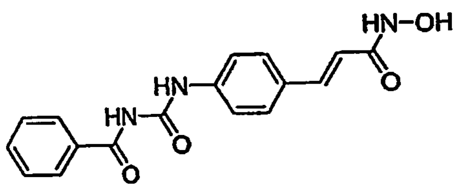
4-(3-Benzoyl-ureidomethyl)-*N*-hydroxy-benzamide



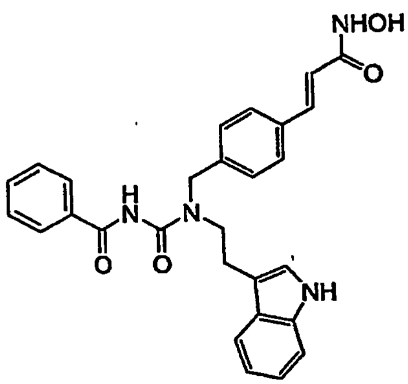
2-[4-(3-Benzoyl-ureido)-phenyl]-*N*-hydroxy-acetamide



2-[3-(3-Benzoyl-ureido)-phenyl]-*N*-hydroxy-acetamide



3-[4-(3-Benzoyl-ureido)-phenyl]-*N*-hydroxy-acrylamide



3-(4-{3-Benzoyl-1-[2-(1*H*-indol-3-yl)-ethyl]-ureidomethyl}-phenyl)-*N*-hydroxy-acrylamide

27. A pharmaceutical composition comprising the compound according to any one of claims 1 through 12.

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